National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

Mutations in the *Broad Complex* affect *dopa* decarboxylase expression at pupariation in *Drosophila melanogaster*.

BY

Michelle Schouls

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

DEPARTMENT OF GENETICS

Edmonton, Alberta

Fall, 1993.

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-88102-X

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Michelle Schouls

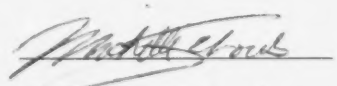
TITLE OF THESIS: Mutations in the *Broad Complex* affect *dopa* decarboxylase expression at pupariation in *Drosophila melanogaster.*

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1993

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.



Department of Genetics

University of Alberta

Edmonton, Alberta

T6G - 2E9

Date: JULY 30/93

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis titled MUTATIONS IN THE *BROAD COMPLEX* AFFECT *DOPA* DECARBOXYLASE EXPRESSION AT PUPARIATION IN *DROSOPHILA MELANOGASTER* submitted by MICHELLE SCHOULS in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

Dr. R. B. Hodgetts, supervisor

Dr. M. Russell

Dr. C. Spencer

Date: July 27, 1993

Abstract

DDC has a complex developmental profile: it seems to be produced in response to a falling 20-OH ecdysone titre at the hatch, the first and second moults, and eclosion, but to a rising one at pupariation. It is also produced in a tissue-specific manner, with approximately 90% of total DDC activity confined to the hypoderm at pupariation and the other 10% present in the central nervous system (Scholnick *et al.*, 1983). Hypodermal *Ddc* regulation at this stage appears to be part of a 20-OH ecdysone triggered gene cascade. The object of this study was to investigate the hypothesis that the *Broad Complex* (*BR-C*), an early 20-OH ecdysone inducible gene (Ashburner *et al.*, 1974; Chao and Guild, 1986), is involved in *Ddc* control in the hypoderm at pupariation. *BR-C* consists of five complementation groups: *br, rbp, 2Bc,* a group that does not complement *br* or *rbp* (*2Bab*), and a non-complementing group (*npr1*) (Kiss *et al.*,1988; C. Bayer, pers. comm.). We have found that mutant alleles of *br*, *npr1* and one allele of *2Bab* drastically reduce DDC activity at pupariation, while activity at the second moult is unaffected. Further, $npr1^{7}$ has no effect on DDC activity in the brain tissue of white prepupae. Competitive PCR analysis of steady-state hypodermal *Ddc* RNA levels at pupariation revealed that *BR-C* mutants which affect DDC activity also show lowered levels of mRNA. The Ashburner model of 20-OH ecdysone action (Ashburner *et al.*, 1974) was formulated to describe the puffing patterns in salivary polytene chromosomes. Our observations suggest that the model may be a more general paradigm of 20-OH ecdysone induced gene cascades in other target tissues.

Acknowledgments

First of all I'd like to thank Ross for your steadfast support and guidance throughout this work. In particular I am grateful your encouragement of my plans for a career in architecture. I'd like to thank Bill C. for your patience, your never-ending helpfulness, for *always* knowing where everything is and, most of all, for hiking all across Philadelphia and San Diego with me and not complaining too much about your aching feet! Timmy, thanks for your friendship during those rough times and thanks for livening up those fly-pushing mornings with your repertoire of dirty jokes and (*yawn*) all those droning men. Thanks, Andrew, for making me computer literate (I couldn't have written this thesis without you!) and for providing me with lots of E-mail entertainment. Millan, thanks for giving me the courage to discover what is really appropriate for me in a career, for teaching me 3-d tetris (wrong!), and for always being my 'hero'. Bill B., thanks for being such a wonderful friend and for giving me a rust-free driver's-side back door! Make them cards bounce! Jana and Andrew, thanks for being more than just technicians but friends! Debra and David, thanks for helping me survive the PSC exam. Vicki and Mark, thanks for always giving me first dibs on the equipment. Thanks, Bill A. for always being a curmudgeon. Thank you, mom and dad, for your constant love and support. And finally, but most importantly, thanks, Janet and Anita, for feeding me!

Table of Contents

I. Introduction 1

II. Materials and Methods 15

Drosophila melanogaster stocks 15

Developmental Staging 17

DDC and Protein assays 17

Genomic DNA Extractions 18

Southern Analysis 19

Preparation of Probes 19

RNA Extractions 20

Reverse Transcription 21

Preparation of the Competitor Plasmid 22

Competitive Polymerase Chain Reaction 22

Computer Analysis of Competitive PCR Gels 23

III. Results 24

Complementation Tests 24

P-element Insertion in br^{28} 27

Dopa Decarboxylase Activity in *BR-C* Mutants 30

Dopa Decarboxylase Activity in the Brains of $npr1^{7}$ and br^{5} Mutants 37

Competitive Polymerase Chain Reaction 38

IV. Discussion 57

Technical Considerations 57

Dopa Decarboxylase Activity in *BR-C* Mutants at the Second Moult 60

Dopa Decarboxylase Activity in White Prepupal Brains of *BR-C* Mutants....61

Dopa Decarboxylase Expression in *BR-C* Mutants at Pupariation....62

Z2 Binds to the First Intron of Ddc....65

Further Studies....67

Conclusions....68

V. Bibliography....70

VI. Appendix 1: DDC Activity in *BR-C* Mutants....75

List of Tables

Table 1: Complementation Tests 26

Table 2: Sample Competitive PCR Calculations 53

Table 3: Steady-State RNA Levels in *BR-C* Mutants 56

List of Figures

Figure 1: Developmental Profile of DDC and Ecdysone....2

Figure 2: The Ashburner Model....6

Figure 3: BR-C Complementation Map....9

Figure 4: Schematic Molecular Map of *BR-C*....11

Figure 5: Crosses for Complementation Tests....25

Figure 6: *Hin*dIII Restriction Map of Z2....27

Figure 7: Southern Showing P-element Insertion in br^{28}....28

Figure 8: Segregation of Genotypes in a Typical Stock....31

Figure 9: DDC Activity in $npr1^{7}$ Mutants....32

Figure 10: DDC Activity in *2Bc* Mutants....33

Figure 11: DDC Activity in rbp^{4} Mutants....34

Figure 12: DDC Activity in *br* Mutants....35

Figure 13: DDC Activity in *2Bab* Mutants....36

Figure 14: Competitive PCR Templates and Primers....39

Figure 15: Gel Showing Competitive PCR Products from Canton S cDNA....44

Figure 16: Gel Showing Competitive PCR Products from rbp^{4} cDNA....46

Figure 17: Gel Showing Competitive PCR Products from br^{8} cDNA....48

Figure 18: Gel Showing Competitive PCR Products from $2Bab^{5}$ cDNA....50

Figure 19: Sample Competitive PCR log-log plots....54

List of Abbreviations

AMV.....avian myeloblastosis virus

ATP.....adenosine triphosphate

bp.....base pairs

br.....*broad*

BR-C.....*Broad Complex*

cDNA.....complementary DNA

CNS.....central nervous system

CPM.....counts per minute

CTP.....cytidine triphosphate

Ddc.....*dopa* decarboxylase (the gene)

DDC.....*Dopa* decarboxylase (the enzyme)

DEB.....diepoxy butane

DEPC.....diethyl pyrocarbonate

DNA.....deoxyribonucleic acid

DTT.....dithiothreitol

EDTA.....ethylenediaminetetraacetic acid

EMS.....ethyl methanesulfonate

GTP.....guanosine triphosphate

kb.....kilobase pairs

M-MLV.....Moloney murine leukemia virus

mRNA.....messenger RNA

npr.....*nonpupariating*

NTP.....any one of ATP, TTP, CTP or GTP

OD.....optical density

PCR.....polymerase chain reaction

rbp.....*reduced bristles on palpus*

RNA.....ribonucleic acid

RNase.....ribonuclease

SDS.....sodium dodecyl sulphate

SSC.....20X = 3M NaCl, 0.3M NaCitrate

TAE.....40mM Tris-acetate, 1mM EDTA

TE.....10mM Tris-Cl, 1mM EDTA

Tris.....Tris(Hydroxymethyl)Aminomethane

tRNA.....transfer RNA

TTP.....thymidine triphosphate

UTP.....uridine triphosphate

UV.....ultraviolet light

y.....*yellow*

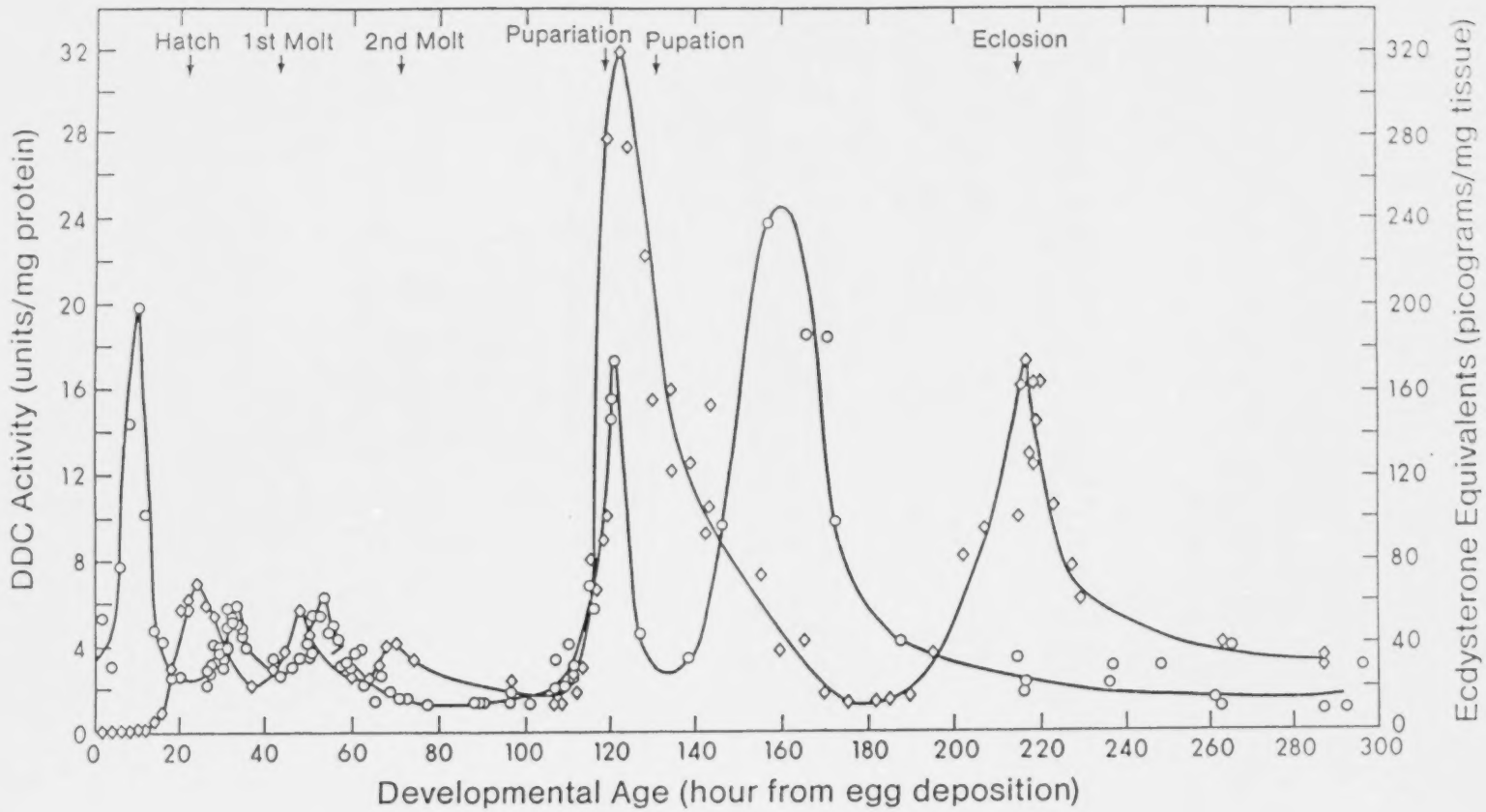
Z1- 4.....zinc finger pairs 1- 4

Introduction

The development of a multicellular organism from a single fertilized ovum requires the orchestration of many genes into a delicate pattern of temporal and spatial expression resulting in growth, programmed cell death and differentiation of tissues. The study of these expression patterns has been greatly aided by the use of model systems amenable to genetic analysis. The fruit fly, *Drosophila melanogaster*, is one such system. It has a fairly complex life cycle, yet its relatively small genome, easily visualized in the polytene chromosomes of the salivary glands, and its short generation time renders it conducive to mutational investigation. Our laboratory is currently studying the *dopa* decarboxylase gene (*Ddc*) in *Drosophila melanogaster*, in an attempt to understand some of the mechanisms involved in its complex developmental expression pattern and tissue specific transcription.

The enzyme *dopa* decarboxylase (DDC) has been localized to the central nervous system (CNS) as well as to the hypoderm (Konrad and Marsh, 1987). It functions in the production of the neurotransmitters dopamine and serotonin in the CNS and in the production of dopamine in the hypoderm from which the quinones essential for the sclerotization of the cuticle are derived (Wright, 1987). While expression of DDC remains at a fairly constant low level throughout development in the CNS (Scholnick *et al.*, 1986), the majority (>90%) of its expression is found in the hypoderm (Lunan and Mitchell, 1969) and fluctuates greatly over the course of the fly's life cycle (Marsh and Wright, 1980; Kraminsky *et al.*,1980; Figure 1). In spite of such a complex developmental expression pattern, there is a single structural gene which specifies DDC (Hirsh and Davidson, 1981).

Figure 1. DDC activity and ecdysone titre during the life cycle of the Canton S strain at 25°C. Enzyme activity is indicated by diamonds and ecdysone titre by circles. This figure has been taken from Kraminsky et al. (1980).


Hatch
1st Molt
2nd Molt
Pupariation
Pupation
Eclosion
DDC Activity (units/mg protein)
Ecdysterone Equivalents (picograms/mg tissue)
Developmental Age (hour from egg deposition)
0
4
8
12
16
20
24
28
32
0
40
80
120
160
200
240
280
320
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Scholnick and co-workers (1983) found that a 7.5 kb *Pst*I fragment which encompasses the gene is sufficient for normal *Ddc* control at the hatch, pupariation and eclosion. This implies that all the sequences necessary to specify much of its complex temporal and spatial regulation are contained within that fragment.

Our laboratory is interested in the temporal control of hypodermal *Ddc* expression. The DDC developmental profile (Figure 1) reveals that a peak of DDC activity is coincident with a pulse of the hormone 20-OH ecdysone (hereafter referred to as ecdysone) at pupariation, but at all other stages there is a significant lag between the ecdysone pulse and the peak of DDC activity. This suggests that ecdysone may be involved in inducing *Ddc* expression at pupariation but not at any other stage in the life cycle. Studies first demonstrating induction of DDC activity by ecdysone were performed on the blow fly, *Calliphora* (Karlson and Sekeris, 1962). These studies revealed that DDC activity increases significantly after injection of ecdysone into isolated *Calliphora* abdomens. Later work by Kraminsky et al. (1980) established that DDC activity at pupariation in the fruit fly is indeed influenced by ecdysone. They found that temperature-sensitive ecd^1 mutants, which are unable to produce high levels of ecdysone at pupariation when held at the restrictive temperature (29°C), were also unable to produce DDC at 29°C. Feeding ecdysone to larvae held at the restrictive temperature, however, resulted in a detectable increase in translatable Ddc mRNA as soon as 2 hours after feeding (Kraminsky *et al.*, 1980).

These results are reminiscent of those of Ashburner *et al.* (1974). Ashburner and co-workers examined the puffing patterns on polytene chromosomes in salivary glands cultured in the presence and absence of ecdysone.

They described 3 different kinds of puffs: a small number of "intermoult" puffs which regressed upon the addition of ecdysone, less than 10 "early" puffs which were rapidly induced by ecdysone and then regressed, and over 100 "late" puffs which responded after a lag period of at least 3 hours following ecdysone administration. Inhibition of protein synthesis by cycloheximide blocked early puff regression and blocked late puff induction. These observations led to the Ashburner model of ecdysone action (Figure 2). This model hypothesizes that ecdysone reversibly binds a receptor protein already present in the salivary glands. This ecdysone-receptor complex represses the intermoult and late genes, but activates the early genes. Once the concentration of early gene proteins is high enough to out-compete the ecdysone-receptor complex, the late genes are activated. Early gene protein products also act as negative regulators for themselves. Thus the early puffs are activated as a primary response to ecdysone, and the late puffs as a secondary response.

This model assumes that puffing indicates transcription, which is not always the case. Puffing and transcription can be uncoupled. For example, most of the transcription of the 68C intermoult puff is due to the glue gene *Sgs-3*, but when *Sgs-3* is inserted into a new site in the genome via transformation, a puff does not occur at that site although the gene is highly transcriptionally active. Also, in an *npr1* mutant background, a prominent puff is still observed at 68C even though no 68C glue gene message accumulation is detected (see Meyerowitz *et al.*, 1987 for a review).

In the hypoderm, the rapid appearance of translatable mRNA is similar to the early puff induction in the salivary glands (Kraminsky et al., 1980). In order to investigate whether *Ddc* induction shares with early gene induction an

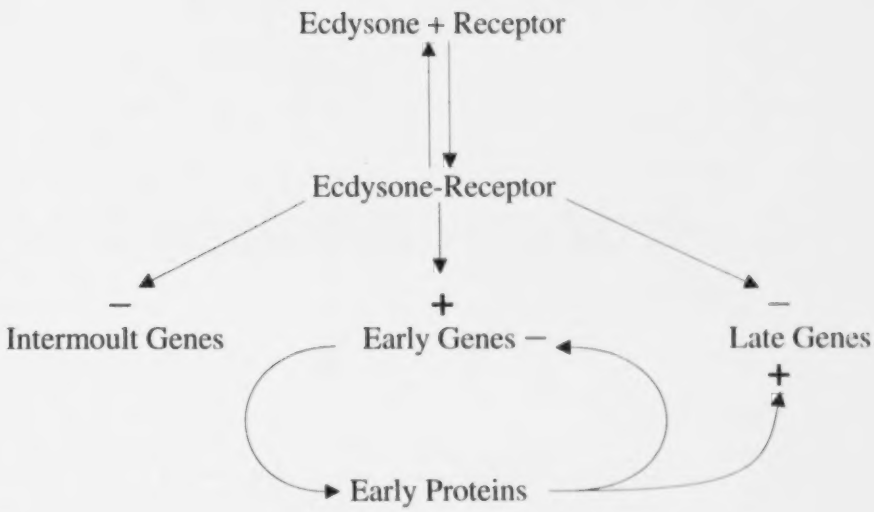


Figure 2. The Ashburner model for the genetic control of polytene chromosome puffing by ecdysone (Ashburner *et al.*, 1974). This figure has been revised from Andres and Thummel (1992).

insensitivity to inhibitors of protein synthesis, Clark *et al.* (1986) expanded the *ecd*^{1} experiments. They fed third instar larvae protein synthesis inhibitors as well as ecdysone at the restrictive temperature. If the larvae were fed only ecdysone, an increase in the steady-state level of *Ddc* transcripts was detected as soon as 2 hours after feeding, similar to an "early" response. Interestingly, the level of *Ddc* transcripts increased 25 fold 6.5 hours after feeding which distinguishes it from the 4 fold increase of 74EF early puff transcripts at this time point. However, if the larvae were fed protein synthesis inhibitors and ecdysone, the response was reduced to a 6 fold increase in *Ddc* transcripts, akin to that of 74EF. Thus, *Ddc* is ecdysone inducible very much like an early gene. But, like a late gene, *Ddc* is also dependent on protein synthesis for its complete range of expression. This result indicates that the regulation of *Ddc* expression has attributes of both early and late gene expression.

Since *Ddc* requires protein synthesis for full expression at pupariation, it may be possible that one of the protein products of the early gene set that is active in the salivary glands mediates *Ddc* induction in the hypoderm. Three of these genes have been defined at the molecular level: *E74* (74EF puff) (Burtis *et al.*, 1990), *E75* (75B puff) (Segraves and Hogness, 1990), and the *Broad Complex* (2B5 puff) (Chao and Guild, 1986; DiBello *et al.*, 1991). These three genes are quite similar. They each encompass approximately 100 kb of genomic DNA, they each have several ecdysone inducible promoters, the mRNA of all three undergoes alternate splicing such that each gene produces several related isoforms, and they all encode site-specific DNA binding proteins. We chose to investigate the *Broad Complex* (*BR-C*) as a candidate for possible involvement in

Ddc regulation at pupariation because it had the most extensive genetic characterization (Belyaeva *et al.*, 1980; Kiss *et al.*, 1988).

BR-C is a very complex locus. It consists of at least 5 complementation groups (Figure 3): *br* (broad; named for the broad wing phenotype of the non-lethal allele, br^1), *rbp* (reduced bristles on the palpus), *l(1)2Bc*, *l(1)2Bab* (which does not complement either *br* or *rbp*) and *npr1* (nonpupariating; alleles of this group do not complement any of the other groups) (Kiss *et al.*, 1988; C. Bayer, pers. comm.). Most *BR-C* alleles are lethal (Kiss *et al.*, 1988) with death occurring at some point between late third instar and formation of the pharate adult (see 'Materials and Methods' for further details). Kiss *et al.* (1988) proposed that *br* and *rbp* may in fact be alleles of the same complementation group since some *br* alleles show *rbp* phenotypes and some *rbp* alleles show *br* phenotypes. In addition, both seem to be involved in elongation and eversion of appendages from imaginal disc, while *2Bc* alleles seem to be involved in the fusion of discs to form a continuous adult epidermis. Later studies have shown, however, that the *br* and *rbp* functions are indeed distinct. Guay and Guild (1991) demonstrated that the *rbp* function is required in the salivary glands for the transcription of 4 intermoult genes and 6 late genes while mutations in the *br* and *2Bc* functions had no effect on the induction of these genes. Restifo and White (1991) found optic lobe disorganization in *br* and *2Bc* mutants but not in *rbp* mutants. Furthermore, *rbp* function is required for programmed cell death of larval salivary glands and for proper thoracic muscle development while *br* and *2Bc* functions are not (Restifo and White, 1992).

Not surprisingly given its genetic complexity, the molecular organization of the *BR-C* locus is very complicated (DiBello *et al.*, 1991). The gene spans

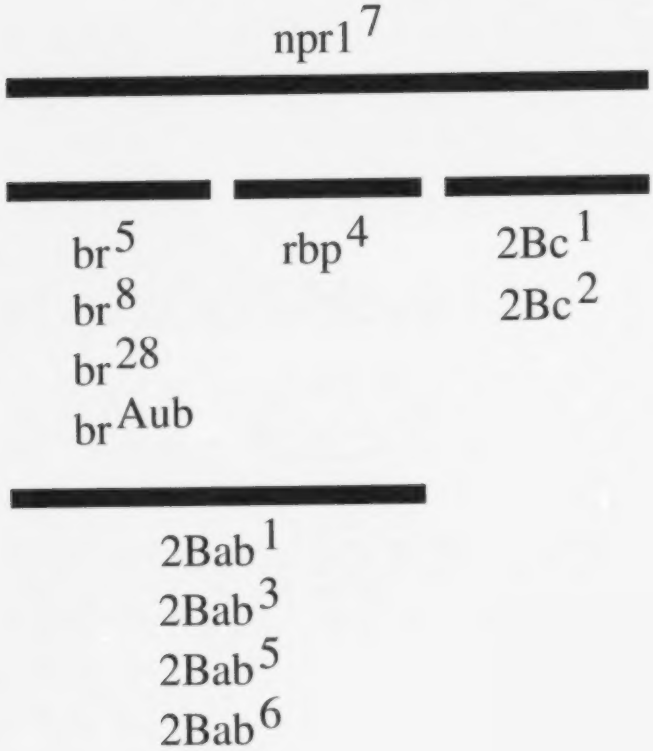


Figure 3. Complementation map of the *Broad Complex* (Kiss *et al.*, 1988; C. Bayer, pers. comm.). The alleles shown are those used in this study.

approximately 100 kb of DNA in the band 2B5. It contains at least three nested promoters whose contiguous exons are alternately spliced to a common core sequence. In turn, the core sequence is spliced to one of three alternative sequences each of which encodes a pair of zinc fingers. Recently, a fourth zinc finger encoding sequence has been discovered (C. Bayer, pers. comm.) (Figure 4). Zinc fingers are DNA binding domains first identified in *Xenopus* TFIIIA (Miller et al., 1985). They are known to assume several configurations (see Evans and Hollenburg, 1988, for a review) and those in *BR-C* are of the type C_2H_2 (DiBello *et al.*, 1991). Attempts to correlate complementation groups with *BR-C* transcripts have had little success because the mutations due to large deletions tend to be uninformative, and most of those due to other smaller lesions have not been mapped at the molecular level. One exception, however, is the br^{28} allele: it is a P-element insertion into Z2 (Sampedro *et al*, 1989; DiBello *et al.*, 1991). This indicates that the *br* complementation group may correspond to Z2 carrying transcripts (DiBello *et al.*, 1991). Genetic mapping has led DiBello *et al.* (1991) to suggest further that Z1 may mediate the *rbp* function and Z3 may mediate the *2Bc* function .

Regulation of *BR-C* is not as straight forward as predicted by the Ashburner model. A small peak of ecdysone activity at 20 hours prior to pupariation seems to induce a subset of *BR-C* transcripts approximately 8 hours later (Karim and Thummel, 1992). Further induction occurs concurrently with the ecdysone peak at pupariation, consistent with the Ashburner model (Karim and Thummel, 1992). The rbp^5 mutation completely blocks transcription of the intermoult glue genes *Sgs-3, Sgs-4, Sgs-5,* and the *71E* gene *VII* (Guay and Guild, 1991), supporting evidence that *BR-C* is expressed prior to early puff induction.

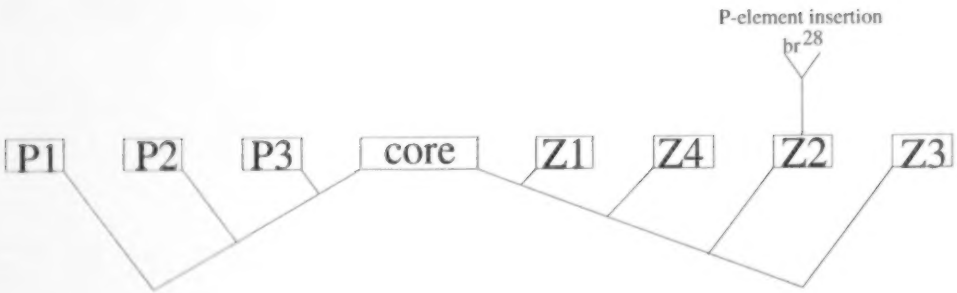


Figure 4. Schematic representation of the alternate splicing pattern of *BR-C* and the approximate insertion site of the P-element in br^{28}. P = promoter and Z = zinc finger pair. For details see DiBello *et al.* (1991).

In addition, Karim and Thummel (1993) found that the *2Bc* function is required for proper intermoult gene repression at pupariation, as well as for maximal induction of the early genes *E74* and *E75*. This corroborates earlier observations of Belyaeva *et al.* (1981) and Zhimulev *et al.* (1982) that the intermoult puff, 68C, failed to regress and the early puffs, 74EF and 75B, were only partially induced in flies carrying a deletion of the 2B5 band or in a $2Bc^{1}$ mutant. This data adds extra dimensions to the Ashburner model. Firstly, not only is the ecdysone-receptor complex required to turn off the intermoult genes at pupariation, but an early gene product is also involved. Secondly, early gene products are involved in positive autoregulation as well as negative. In further support of the second point, Karim and Thummel (1993) reported that the *br* function may be involved in positive regulation of the smallest *BR-C* transcript produced at pupariation. Also, L. VonKalm *et al.* (pers. comm.) suggested that the *2Bc* function may act as a positive control on *BR-C* because expression of Z1 and Z3 transcripts is delayed by up to 16 hours in $2Bc^{1}$ salivary glands. In contrast, they suggest that the *rbp* function may act as a negative control because the Z1 and Z3 transcripts, although they appear on time, are overexpressed in rbp^{5} mutants.

BR-C is not only required in the salivary glands, but in several other tissues as well. However, while *BR-C* is expressed during late third instar and pupariation in the salivary glands, its effects in other tissues seems to be limited to metamorphosis. As described earlier, the *br*, *rbp* and *2Bc* functions are all necessary for wild type imaginal disc morphogenesis (Kiss *et al.*, 1988). The *br* function appears to regulate the expression of *Stubble-stubbloid* (*Sb-sbd*) and of *blistered* (*bs*) in discs (Gotwals and Fristrom, 1991). Alleles of *npr1* cause reduction in expression of the fat body protein *Larval Serum Protein-6* (*LSP-6*)

and fail to prevent repression of the gene at high ecdysone concentrations (Nelson *et al.*,1991), which normally occurs. The *br* and *2Bc* functions are necessary at metamorphosis for proper positioning and organization of the optic lobes, a process under hormonal control (Restifo and White, 1991), and also for normal shape and location of the proventriculus (Restifo and White, 1992). Midgut transition requires wild type *2Bc* function; programmed cell death of the larval salivary glands and normal flight muscle development require *rbp* function (Restifo and White, 1992).

It is interesting that recent studies using reverse transcription-PCR have shown distinct differences between tissues in terms of which zinc finger bearing *BR-C* transcripts are expressed (Huet *et al.*,1993). For example, Z1 and Z3 are present at much higher levels than Z2 in the salivary glands where *rbp* and *2Bc* are involved in intermoult, early and late gene control. Z2 is more prevalent than Z3 in wing discs where the *br* function plays an important role in wing morphogenesis. These differences strengthen the correlation between the zinc fingers and the complementation groups suggested by DiBello *et al.* (1991).

BR-C, then, is a key locus in ecdysone induced gene cascades. Its function is proving to be even more complex than originally predicted by the Ashburner model. Its transcriptional response occurs not only in response to the ecdysone peak at pupariation, but also to the previously undetected small peak at mid-third instar (Karim and Thummel, 1992) and to the larger mid-pupal peak (Andres *et al.*, 1993; Huet *et al.*, 1993). It acts as a positive and negative regulator of its own expression and that of many salivary gland genes expressed at pupariation, as well as for several genes expressed in other tissues during metamorphosis. The question remains as to whether *BR-C* function in tissues other than the salivary

glands is limited to metamorphosis, or if it is also required at pupariation in other target tissues. Since *BR-C* transcripts have been detected in wing discs, malpighian tubules, the fat body and the gut at pupariation (Huet et al., 1993), one might expect that *BR-C* function is necessary at this stage in these tissues.

The role of *BR-C*, if any, in the hypoderm at pupariation has not previously been addressed. *Ddc*, like *BR-C* and the other early genes described by the Ashburner model, is rapidly induced by ecdysone. However, in order to attain full transcriptional expression, it requires protein synthesis (Clark *et al.*, 1986). These findings led us to search for a gene whose product could be involved in positively regulating *Ddc* at this stage. *BR-C*, with its complex genetics, and its battery of DNA binding protein products (Kiss *et al.*, 1988; DiBello *et al.*, 1991), seemed a logical candidate to pursue. The purpose of this study was to ascertain whether *BR-C*, and in particular whether one of its subfunctions, played a role in mediating full *Ddc* expression in the hypoderm at pupariation.

Materials and Methods

Drosophila melanogaster Stocks

Drosophila melanogaster chromosomes used in various strains (Lindsley and Zimm, 1992):

Binsn - an X chromosome balancer marked with the dominant mutation *Bar* and the recessive mutation *singed* (In(1)sc^{S1}L sc^{8}R+dl-49, sc^{S1} sc^{8} sn^{X2} B). Males are viable and fertile, homozygous females are viable but sterile.

FM6 - an X chromosome balancer marked with the dominant mutation *Bar* and the recessive mutation *yellow* (In(1)FM6, y^{31d} sc^{8} dm B). Males are viable and fertile, homozygous females are viable but sterile.

X^X,yf - an attached X chromosome marked with the recessive mutations *yellow* and *forked.*

Y (dup) - a Y chromosome carrying a duplication for the *BR-C*: y^{2}Y67g19.1. This was used for complementation testing.

y npr1^{7} - *npr1*^{7} is a DEB induced mutation, resulting in a translocation between chromosomes 1 and 3 (T(1;3)2B5;61F3-4) and subsequently is null for the *BR-C* (Kiss et al. 1988). It is an amorphic allele, hemizygous males fail to pupariate but survive 10-15 days after their normal sibs have pupariated.

y l(1)2Bab^{1} - *l(l) 2Bab*^{1} is an hypomorphic allele, hemizygous males arrest late in the pupal stage. It is an EMS induced mutation (Kiss et al. 1988).

y l(1)2Bab^{3} - *l(l)2Bab*^{3} is very similar to *l(1)2Bab*^{1}(Kiss et al. 1988).

y l(1)2Bab^5 - *l(l)2Bab^5* is the strongest *2Bab* allele, it is a DEB induced mutant (Cindy Bayer, pers. comm.).

y l(1)2Bab^6 - *l(l)2Bab^6* is stronger than *2Bab^1*, but weaker than *2Bab^5*. It is EMS induced (Cindy Bayer, pers. comm.).

y rbp4 - *rbp^4* an hypomorphic allele, hemizygous males arrest as late pupae, most reaching the pharate adult stage. It is an EMS induced mutation (Belyaeva, 1980).

y l(1)2Bc^1 - *l(l)2Bc^1* is an amorphic allele, hemizygous males arrest as either late prepupae or as early pupae. It is EMS induced (Kiss et al. 1988).

y l(1)2Bc^2 - *l(l)2Bc^2* is very similar to *l(l)2Bc^1* (Kiss et al. 1988).

y br^5 - *br^5* is an amorphic allele, hemizygous males arrest as untanned pupae. It is an EMS induced mutation (Kiss et al. 1988).

y br^8 - *br^8* is very similar to *br^5* (Kiss et al. 1988).

br^{28} - *br^{28}* is very similar to *br^5*, but stronger in phenotype. This allele is caused by a P-element insertion in the Z2 zinc finger of the *BR-C* (DiBello et al. 1991).

br^{Aub} - *br^{Aub}* is a weak hypomorphic allele, hemizygous males are viable. It was found in the wild by Mel Green (Cindy Bayer, pers. comm.).

Drosophila melanogaster strains:

Canton S - a standard wild type laboratory strain.

X^X,yf / Y(dup) - an attached X strain, in which the Y chromosome carries a duplication for the *BR-C* (see previous description). This strain was the source of the Y(dup) chromosome used in the complementation tests.

All chromosomes carrying a *BR-C* mutation were maintained in stocks by balancing them over either Binsn (if the chromosome was marked with *yellow*), or FM6 (if the chromosome was *yellow*$^+$).

All organisms were maintained on standard fly food (Nash and Bell, 1968) at 22°C.

Developmental Staging

Organisms were staged and collected as described in Patel (1992). Collections were made at the second moult and at the white prepupal stage. The organisms were sexed and only males were chosen. *Yellow* (*y*) males were then separated from wild type based on the pigmentation of the dentical belts and mouth parts and were frozen at -70°C. For DDC assays, 6 - 10 newly moulted third instars of each genotype were frozen away, and 4 white prepupae of each genotype. For RNA extractions, 6 -10 white prepupae of each were collected and frozen. All of these collections were done on at least two separate occasions.

DDC and Protein Assays

DDC assays (Walter *et al.* 1991) were performed on organisms frozen for less than one month. Newly moulted third instars were homogenized at a concentration of one organism in 20µl of buffer. White prepupae were homogenized at 50µl per organism. Brains dissected from white prepupae were

homogenized at 5µl per brain. For each extract, three 5µl aliquots were assayed with a typical standard deviation of 3%. Assays were done in duplicate or triplicate on all genotypes, each assay on organisms collected on separate occasions.

Protein determinations were made using the Coomassie Protein Assay Reagent kit from Pierce. Each homogenate was diluted 1:100 in triplicate and assayed. DDC activities were not corrected for protein concentration for reasons outlined by Patel (1992); rather they were normalized on a per organism basis.

Genomic DNA Extractions

For each extraction, 30 flies were collected and frozen at -70°C. These were homogenized, in a glass tissue grinder, in 500µl of 100mM NaCl, 100mM Tris-Cl pH 8.0, 50mM EDTA and 2.5 µl of 10mg/ml RNase A. The homogenate was incubated for 10 minutes at room temperature, after which 2.5µl of 20mg/ml proteinase K was added and it was incubated for 1-2 hours at 65°C with occasional inversion of the tube. The debris was removed by microcentrifugation for 1-2 minutes and the supernatant was extracted with 500µl Tris-saturated phenol. The aqueous layer was extracted once more with 250µl each of phenol and chloroform, and once again with 500µl of chloroform. Cold 95% ethanol (1.5 volumes) was added to precipitate the DNA. It was pelleted by 10 minutes of centrifugation in the microfuge. The pellet was washed in 70% ethanol, dried, and resuspended in 100µl of TE, pH 8.0. The phenol/chloroform extractions were repeated, the DNA was precipitated (after adding NaAcetate to 0.3 M),

pelleted, washed, dried, and resuspended in 100µl of TE. The precipitation was carried out once more to remove any excess salt.

Southern Analysis

Genomic DNA was digested for four hours, then subjected to electrophoresis on a 0.8% agarose gel made with TAE for 20 hours at 27 volts. The DNA was transferred to a nylon membrane (Gene Screen from Du Pont) using southern transfer and UV crosslinked to it for 1 1/2 minutes. The blot was prehybridized for two hours in the following solution: 50% formamide, 1% SDS, 1M NaCl, and 0.2 mg/ml yeast tRNA. Riboprobe was added to a final concentration of 10^6 CPM/ml and allowed to hybridize overnight. The blot was washed twice in 2X SSC for five minutes at room temperature, twice in 2X SSC, 1% SDS for 30 minutes at 60°C and once in 0.1X SSC for 30 minutes at room temperature. It was then exposed to Fuji Medical X-ray film for 48 hours, and the autoradiograph was developed for two minutes.

Preparation of Probes

Riboprobes (Melton *et al.*, 1984) are often generated using a plasmid containing an RNA polymerase T3 promoter flanking one side of the insert of interest and an RNA polymerase T7 promoter flanking the other. However, in this thesis the plasmid was pCR 1000, which only contains the T7 promoter. The

plasmid was linearized at the appropriate site, and 200ng of it was added to 4µl of 5X transcription buffer (0.2M Tris-Cl pH 8.0, 0.125M NaCl, 40 mM $MgCl_2$, 10 mM spermidine), 1µl of 100mM DTT, 1µl RNAguard (Pharmacia), 3µl NTP solution (1.5 mM each of ATP, GTP and CTP), 9 µl of [α-^{32}P] UTP (10µCi/µl) and 1µl of the appropriate RNA polymerase. The reaction was allowed to proceed for 1 hour at 37°C. The labeled probe was separated from unincorporated counts by passing the reaction over a Sephadex G-50 column.

RNA Extractions

RNA extractions were carried out as per Thummel (pers. comm.). Six to ten white prepupae were placed in a glass tissue grinder in 400µl of RNA extraction buffer (0.1 M Tris-Cl pH 8.2, 0.1 M NaCl, 20 mM EDTA, 1% Sarcosyl) and homogenized with one stroke. Immediately, 400µl of Tris-saturated phenol was added and the organisms were homogenized 10 more strokes or until an even suspension was obtained. The homogenate was transferred to an eppendorf tube and centrifuged for 5 minutes to separate the phases. The aqueous phase was extracted once more with phenol, or until the interphase disappeared. It was then extracted with water-saturated ether three times. Cold 95% ethanol (2.5 volumes) was added and the mixture was allowed to sit at room temperature for >30 minutes. The RNA was pelleted by centrifugation at 15,000 X g for 5 minutes at room temperature. The pellet was dried and resuspended in 20µl of DEPC treated sterile water. In order to measure the concentration, 4µl of this was diluted into 1ml of DEPC treated sterile water and its OD_{260} was read on the

spectrophotometer. The remaining RNA was stored at -70°C for later use. RNA extractions were performed in duplicate on each strain.

Reverse Transcription

The RNA (100ng) was heated to 90°C for 5 minutes, quick chilled on ice and microfuged briefly. On ice, 1mM dithiothreitol, 32 units RNAguard (Pharmacia), 22 units reverse transcriptase (AMV, Boehringer Mannheim), and 1X reverse transcription buffer were added to the RNA to a total volume of 40µl. A 10X stock of reverse transcription buffer was made ahead of time containing: 500mM Tris-Cl pH 8.2, 15mM $MgCl_2$, 500mM KCl, 0.01% gelatin, 5mM dNTPs (each), 0.01mM primer. The reaction was incubated for 60 minutes at 42°C. It was then heated to 95°C for 5-10 minutes, quick chilled on ice, and frozen at -70°C.

Other reverse transcriptases were tried, but found to be unsatisfactory. The cDNA produced by M-MLV reverse transcriptase (BRL), when subjected to PCR consistently produced an unexpected product. This product, when sequenced and sent to Gen Bank data base at the National Research Council for a homology search was not found to be homologous to any currently known *Drosophila* sequences. The cDNA produced by AMV reverse transcriptase (Seikagaku America, Inc.) tended to result in blurred bands when subjected to PCR.

Preparation of the Competitor Plasmid

Two double stranded oligonucleotides (MIS4 and 5,and MIS 6 and 7; Figure 14, C) were synthesized (DNA synthesis facility, department of Microbiology, U of A) such that their overhanging ends facilitated ligation to a 472 bp fragment of Ddc DNA and insertion into pUC 19. They acted as linkers between the Ddc DNA and pUC 19 and facilitated annealing of the primers(MIS 1 and MIS 3) in competitive PCR reactions. The oligonucleotides, the Ddc DNA and pUC 19 were ligated by adding equimolar amounts of each, 1X ligation buffer (from a stock of 5X BRL buffer) and 1.5 units of T4 DNA ligase (BRL) in a total volume of 33µl at 15^{o}C overnight. Transformation was carried out using DH5α competent cells as per Sambrook *et al.* (1989). The cells were plated on MacConkey / lactose / ampicillin (100µg/ml) medium. Positive white colonies were streaked out on fresh plates, and mini-preparations of plasmid DNA were done using the Magic Miniprep kit from Promega. The DNA concentration was determined by diluting 5µl of DNA in 1ml of sterile water and reading its OD_{260} on a spectrophotometer.

Competitive Polymerase Chain Reaction

A PCR master mix was made up containing 1X reaction mix (from a 10X stock: 500mM Tris-Cl pH 8.2, 15mM $MgCl_2$, 500mM KCl, 0.01% gelatin, 2mM dNTPs(each), 2µM primers (each)), the frozen 40µl reverse transcription mix containing the cDNA, and 5µl of Taq polymerase in a total volume of 155µl. A

series of 9 small eppendorf tubes, each containing 15µl of sterile water and a different concentration of competitor plasmid DNA (ranging from 0.00125 amol to 20.48 amol, quadrupling each time) was set up. To each tube, 15µl of the PCR master mix was added. Paraffin oil was then layered over each reaction mixture. PCR was carried out as follows: 1 cycle of 95°C for 3 minutes, 62°C for 1 minute, 74°C for 2 minutes; and 29 cycles of 94°C for 1 minute, 62°C for 1 minute,74°C for 2 minutes. This protocol is adapted from Gilliland *et al.* (1990). All PCR was performed on a Stratagene RoboCycler 40. From each reaction mixture 25µl was removed and electrophoresed on a 2% TAE agarose gel at 100 volts for 1-2 hours. Gels were photographed on a Gel Print 2000i video imager and stored for later computer analysis.

Computer Analysis of Competitive PCR Gels

Bands were quantified using the Gel Print Toolbox version 2.0 computer program. This program graphs bands as peaks of ethidium bromide staining and provides integrals of peak areas.

Results

Complementation Tests

All of the Broad Complex mutant strains were sent to us from the Fristrom laboratory. In order to verify the current complementation map (Figure 3), complementation tests were carried out on all strains. The strains $2Bab^{6}$ and br^{Aub} were not tested as they were received very late in this study. The crosses were designed as illustrated in Figure 5. Since males carrying a *BR-C* mutation die before reproductive maturity, the males used in these crosses carried a duplication of the wild type *BR-C* locus on the Y chromosome. For each cross, approximately 200 flies were scored and the results are shown in Table 1, A. The br^{28} strain was not tested against itself in Table1, A because initially the strain carried a second site lethal on the X chromosome. Crosses were undertaken to generate a lethal-free chromosome. Although it was eventually obtained, a lethal-free br^{28} strain became available from the Fristrom laboratory and this strain, rather than ours, was used for further analysis since it had been more fully characterized. It was then tested against $2Bab^{1}$, $2Bab^{3}$, and $2Bab^{5}$ which had all been sent to us at the same time (Table 1, B). No contradictions between the data in Table 1 and the complementation map shown in Figure 3 were observed.

A.

$$\frac{y\ BR\text{-}C(1)^-}{Y\ (dup)} \times \frac{y\ BR\text{-}C\ (2)^-}{Binsn}$$

$$\downarrow$$

$$\frac{Binsn}{Y\ (dup)} \qquad \frac{y\ BR\text{-}C(2)^-}{Y\ (dup)} \qquad \frac{Binsn}{y\ BR\text{-}C(1)^-} \qquad \frac{y\ BR\text{-}C(2)^-}{y\ BR\text{-}C(1)^-}$$

Score *yellow* females for complementation.

B.

$$\frac{y\ BR\text{-}C(1)^-}{Y\ (dup)} \times \frac{BR\text{-}C(2)^-}{FM6,y}$$

$$\downarrow$$

$$\frac{FM6,y}{Y\ (dup)} \qquad \frac{BR\text{-}C(2)^-}{Y\ (dup)} \qquad \frac{FM6,y}{y\ BR\text{-}C(1)^-} \qquad \frac{BR\text{-}C(2)^-}{y\ BR\text{-}C(1)^-}$$

Score *yellow*$^+$ females for complementation.

Figure 5. Crosses for complementation tests where BR-C(1)$^-$ and BR-C(2)$^-$ represent any two *BR-C* alleles. (A) shows the scheme used when Binsn was the X chromosome balancer, and (B) the scheme when FM6 was the balancer.

A.

X	npr1^{7}	2Bc^{1}	2Bc^{2}	rbp^{4}	br^{5}	br^{8}	br^{28}
npr1^{7}	—						
2Bc^{1}	—	—					
2Bc^{2}	—	—	no progeny				
rbp^{4}	—	+	+	—			
br^{5}	—	+	+	+	—		
br^{8}	—	+	+	+	—	—	
br^{28}	—	+	+	+	—	—	not tested

B.

X	2Bab^{1}	2Bab^{3}	2Bab^{5}
br^{28}	—	—	—

Table 1. Complementation tests between various BR-C alelles. The tests shown in (A) were completed at an earlier date than the tests shown in (B).

P-element Insertion in *br*^{28}

In order to confirm that the *br*^{28} strain sent to us still carried the 1.2 kb P-element insertion in Z2 as originally described (Sampedro et al.,. 1989), it was investigated via southern analysis. Genomic DNA was isolated from heterozygous br^{28}/FM6 females and digested for 3 hours with *Hin*dIII (BRL). It was then subjected to a southern analysis as described in "Materials and Methods". A riboprobe was generated from pCR 1000 containing a 197 bp Z2 fragment which encompasses the *Hin*dIII site 36 bp from the P-element insertion in *br*^{28} (Figure 6). If there was no P-element insertion, only two bands were expected: a 3.7 kb band and a 2.3 kb band. If there was an insertion, three bands were expected: the two wild type bands (3.7 kb and 2.3 kb) contributed by the FM6 chromosome, and an extra 4.9 kb band (from the 3' genomic *Hin*dIII site to the *Hin*dIII site in the P-element) from the mutant chromosome. The tiny internal *Hin*dIII fragment released from the mutant chromosome would be too small to detect and in any case has only 36 bp of homology to the probe. The results were as expected confirming that our *br*^{28} strain does indeed still retain the 1.2 kb P-element insertion (Figure 7).

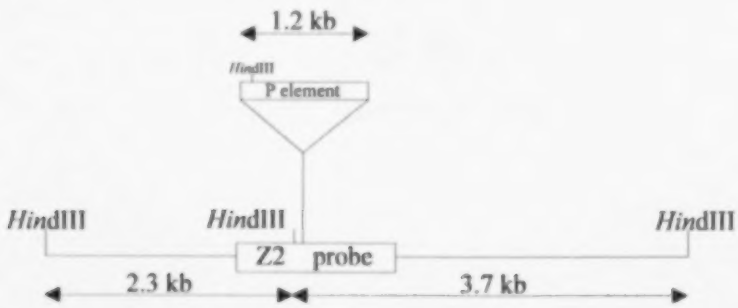
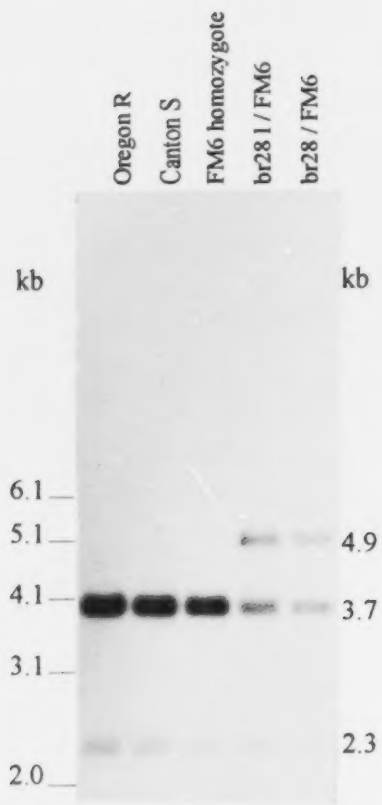


Figure 6. *Hin*dIII restriction map of the Z2 region of the *BR-C* showing the region of homology to the probe used.

Figure 7. Autoradiograph of a southern blot of genomic DNA digested with *Hin*dIII and probed with a riboprobe specific to Z2. DNA from the br^{28} strain carrying the second site lethal was run in the fourth lane, and DNA from the lethal-free strain was run in the fifth.


Oregon R
Canton S
FM6 homozygote
br28 1 / FM6
br28 / FM6
kb
kb
6.1
5.1
4.1
3.1
2.0
4.9
3.7
2.3

Dopa Decarboxylase Activity in *BR-C* Mutants

The hypothesis that Ddc expression is controlled in part by gene products of the BR-C at pupariation, but not at any other stage, was tested by quantifying DDC activity in BR-C mutants at pupariation and at the second larval moult. The second moult was chosen as a stage representative of all the other stages in the life cycle where ecdysone does not play an immediate role in the activation of *Ddc* transcription. Organisms were collected and assayed as described in "Materials and Methods". Since most *BR-C* mutants die in early to mid-pupal stages, hemizygous males could be collected at both the second moult and pupariation. An exception to this is $npr1^{7}$, where the mutants do not pupariate at all. In this case, mutant male wandering third instar larvae were collected at the same time that their wild type siblings were pupariating. In presenting the results, wild type males segregating in the same stock (Figure 8) were used as a standard; that is, DDC activity of mutant males was taken as a percentage of DDC activity of wild type males. Mutant and wild type males were distinguished by the marker *yellow (y)* which can be scored by mouth hooks and denticle belts.

The data are shown in Appendix 1 and summarized in Figures 9 - 13. Organisms assayed at the second moult showed no significant difference amoung mutant strains when subjected to one way analysis of variance or to Duncan's multiple-range test (Snedecor and Cochran, 1980). However, one way analysis of variance showed a highly significant ($p<0.01$) difference amoung mutant strains at pupariation. Duncan's multiple-range test revealed four significantly different groups of mutant strains. Group A consists of br^{Aub} only, a viable *br* mutation, which shows no reduction in activity but, in fact, a slight increase. The second

group, B, contains all those which show activity equivalent to wild type: *rbp*^{4}, *2Bc*^{1}, *2Bc*^{2}, *2Bab*^{1}, *2Bab*^{3} and *2Bab*^{6}. Group C is *br*^{8}, which has about half of wild type DDC activity. Finally, *br*^{5}, *br*^{28}, *npr1*^{7} and *2Bab*^{5}, which all show a severe reduction in activity, make up group D. There is a strong correlation between mutations in the *br* complementation group (except for *br*^{Aub}) and reduced DDC activity. The only other strains which exhibit a significant reduction in DDC activity are those carrying *npr1*^{7} and *2Bab*^{5}. Since *npr1*^{7} does not complement any of the other groups and is a null for the locus, this was to be expected. The *2Bab* group which complements neither *rbp* nor *br* might also be expected to reduce DDC. However, only one allele of the four tested demonstrated any significant reduction.

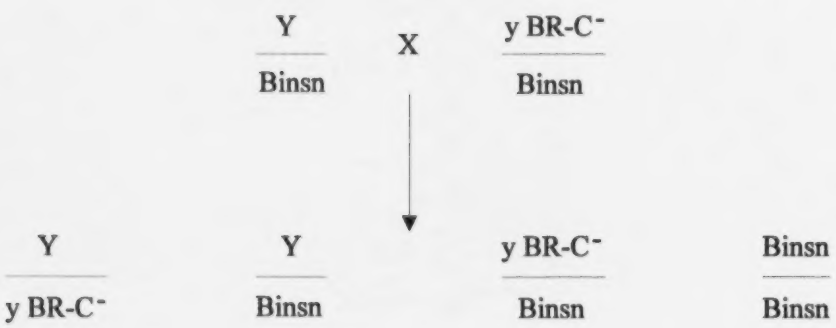


Figure 8. Segregation of genotypes in a typical stock where BR-C^{-} represents any *BR-C* allele. If the stock was balanced over FM6, then the males carrying the *BR-C* mutation were *yellow*$^{+}$, and the wild type males were *yellow*$^{-}$.

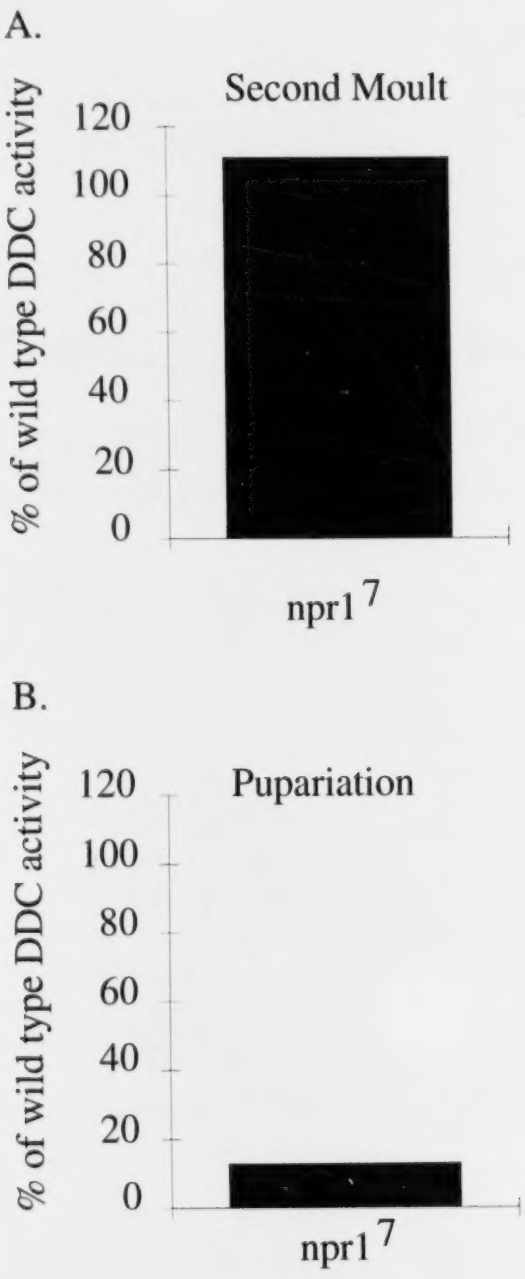


Figure 9. Mean DDC activity in npr1 ^{7} males, expressed as a percentage of the activity of wild type siblings segregating in the same cross, and shown at two stages in development: A) the second moult, and B) pupariation.

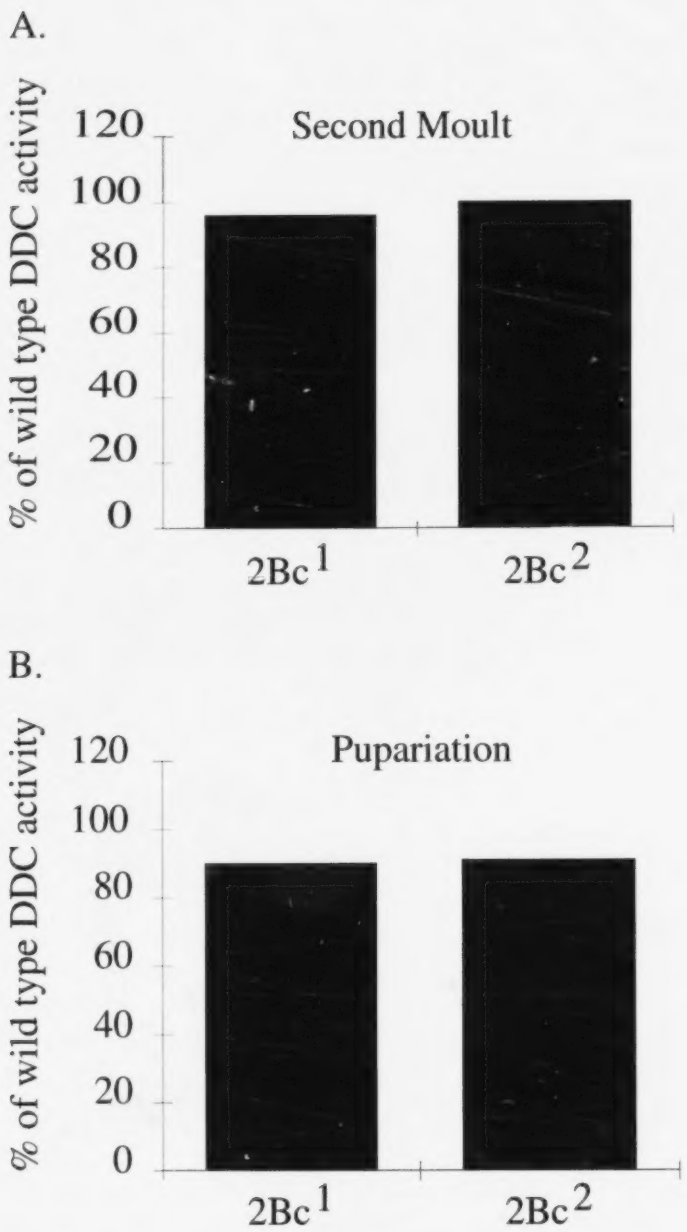


Figure 10. Mean DDC activity in males carrying alleles of the 2Bc complementation group, expressed as a percentage of the activity of wild type siblings segregating in the the same cross and shown at two stages in development: A) the second moult, and B) pupariation.

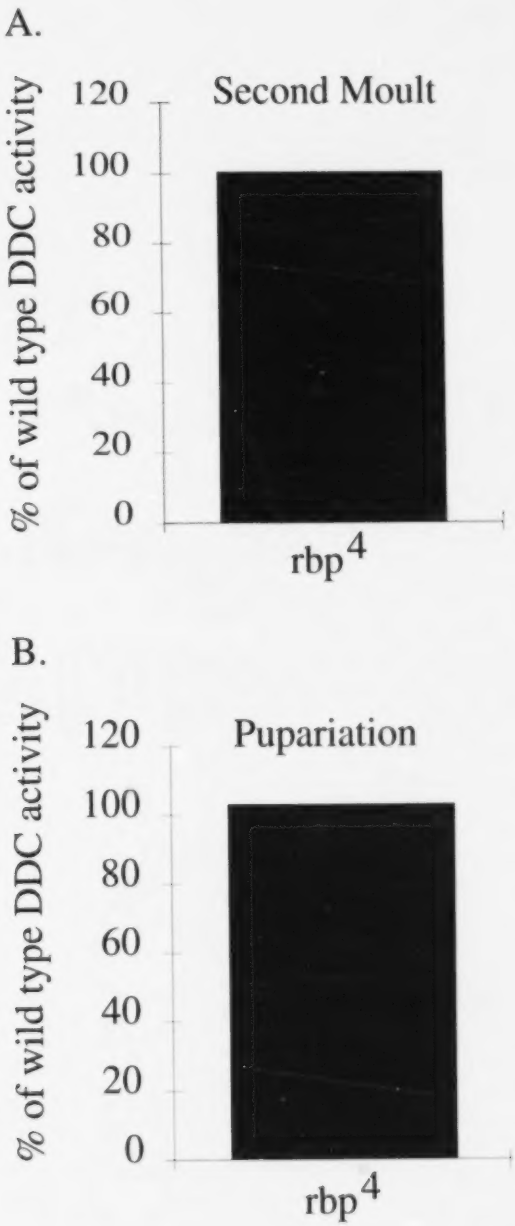


Figure 11. Mean DDC activity in rbp4 males, expressed as a percentage of the activity of wild type siblings segregating in the same cross, and shown at two stages in development: A) the second moult, and B) pupariation.

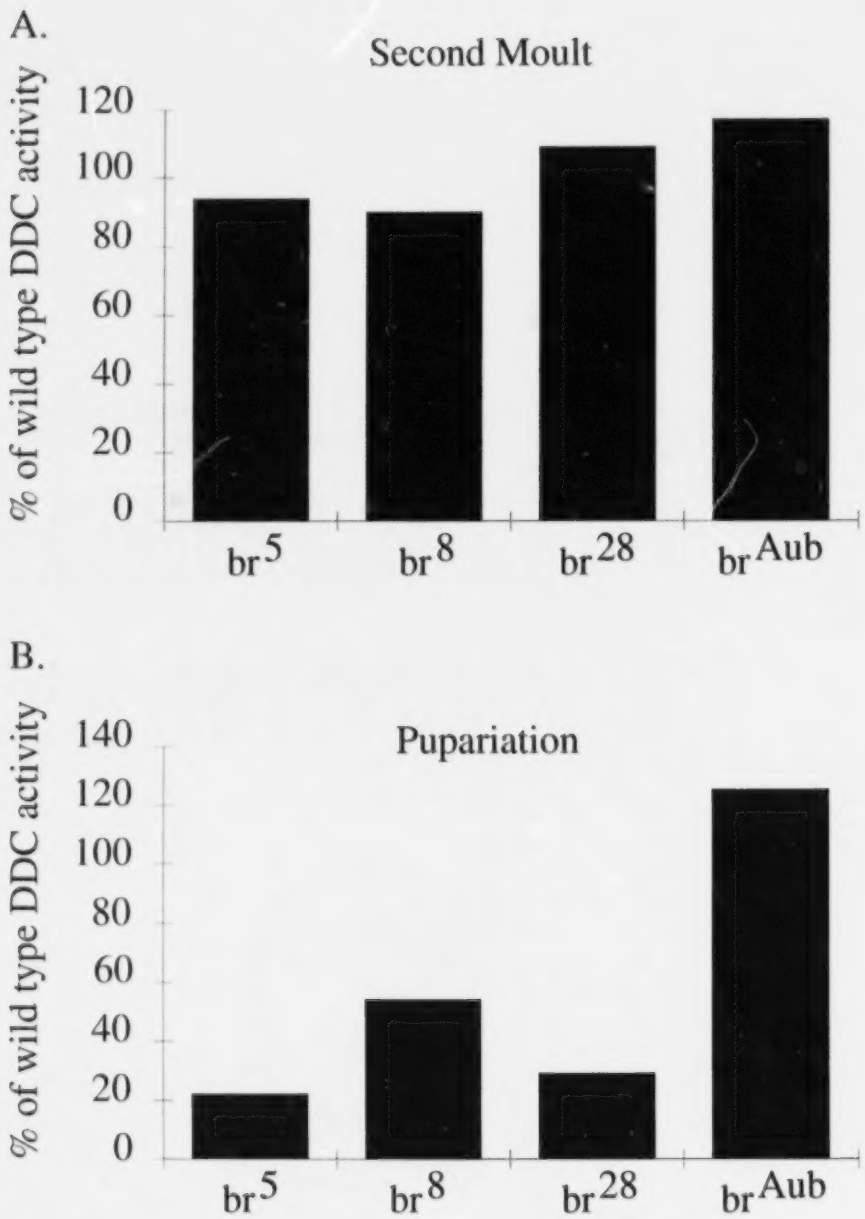


Figure 12. Mean DDC activity in males carrying alleles of the br complementation group, expressed as a percentage of the activity of wild type siblings segregating in the same cross, and shown at two stages in development: A) the second moult and, B) pupariation.

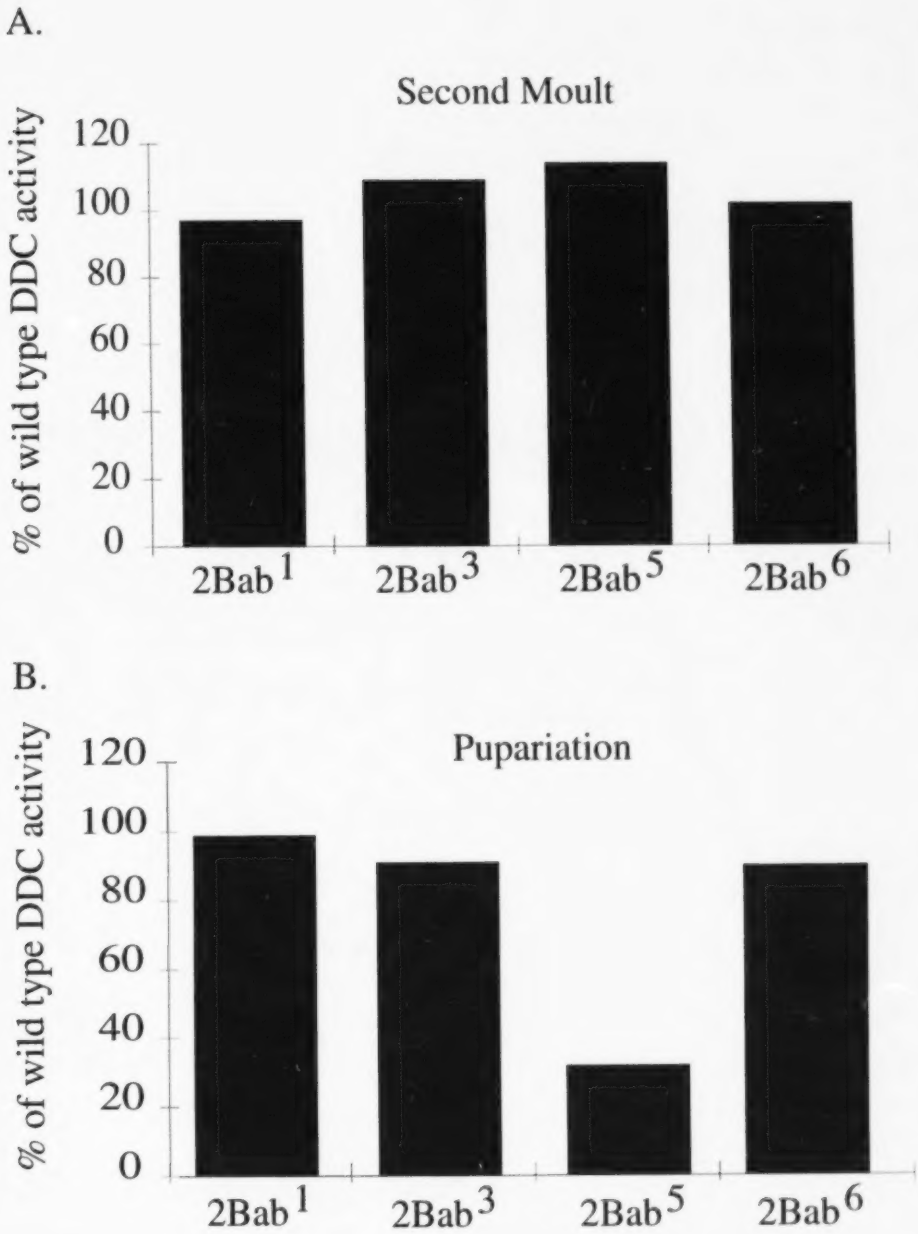


Figure 13. Mean DDC activity in males carrying alleles of the 2Bab complementation group, expressed as a percentage of the activity of wild type siblings segregating in the same cross, and shown at two stages in development: A) the second moult, and B) pupariation.

Dopa Decarboxylase Activity in the Brains of *npr1*^{7} and *br*^{5} Mutants

To ensure that any reduction in DDC activity found in *BR-C* mutants is due to hypodermal DDC reduction and not to reductions in the central nervous system, brains from 2 of the mutants were dissected and assayed. The *npr1*^{7} allele was chosen in particular since, of all the mutants, it showed the greatest reduction of DDC activity at pupariation. Brains from hemizygous *npr1*^{7} males were dissected, collected in triplicate, and assayed for DDC activity. The activity was expressed as a percentage of the activity of wild type brains from males segregating in the same cross. From the 3 separate collections the activity was 117%, 110%, and 132% (mean = 120% ± 9%). One assay on brains collected from *br*^{5} mutant males yielded 111% of the activity of brains from wild type males segregating in the same cross.

Competitive Polymerase Chain Reaction

Once it had been established that there was a correlation between certain mutant alleles of the *BR-C* and a decrease in DDC activity, the question arose as to whether the differences seen in the enzyme levels were reflected in the steady-state levels of mRNA. In order to address this question, levels of *Ddc* mRNA in $npr1^{7}$, $2Bab^{5}$, and all the *br* mutants were quantified using competitive PCR (Gilliland *et al.*, 1990). This technique was chosen as it is highly sensitive and can detect very small amounts of mRNA, circumventing difficulties in collecting large numbers of organisms from which to extract RNA.

The principle behind the technique is that all templates present in a PCR reaction mixture will compete for available substrates. Two templates are used which require the same primer pair for amplification but which produce products that are distinguishable by their size. One template is the unknown amount of *Ddc* cDNA, reverse transcribed from a specific quantity of total RNA. The other is a known amount of plasmid DNA, engineered for the purpose of competing with the cDNA template. A series of PCR reactions is performed, each containing a constant (but unknown) amount of reverse transcribed RNA and a differing known amount of competitor. The reaction which produces equal amounts of competitor and cDNA products (i.e. a ratio of 1) is that in which the amount of template cDNA is equal to the amount of competitor added. A potential difficulty is that differing sizes of the two products may be a cause of differing amplification efficiency; however, if the size difference is kept to a minimum this problem is negligible (Gilliland *et al.*, 1990).

Figure 14. A. The sequence of the 2 *Ddc* primers used in the competitive PCR.

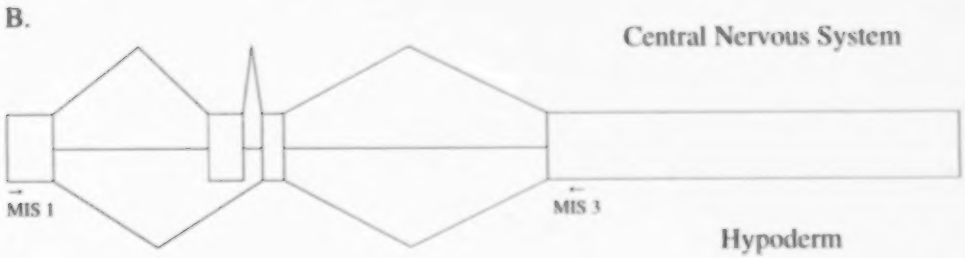
B. Schematic representation of the *Ddc* gene. Boxes represent exons and straight lines represent introns. Peaked lines show the differential splicing patterns in 2 tissues: the central nervous system and the hypoderm. Arrows indicate where the primers (MIS 1 and MIS 3) anneal, arrow heads indicate the 3' ends. The scale is approximately 1mm = 26 bp (the size of the primer arrow is exaggerated so that it is more easily seen).

C. Schematic representation of the competitor plasmid, pMS1. Arrows indicate where the primers used in PCR (MIS 1 and MIS 3) anneal, arrow heads indicate the 3' ends. The oligonucleotides MIS 4, 5, 6 and 7 were used in the construction of pMS1. MIS 5 is identical to MIS 1, with overhanging restriction site ends as shown. MIS 4 is the complement of MIS 5 with its overhanging ends as shown. MIS 6 is identical to MIS 3, with overhanging restriction site ends as shown. MIS 7 is its complement with its overhanging ends as shown.

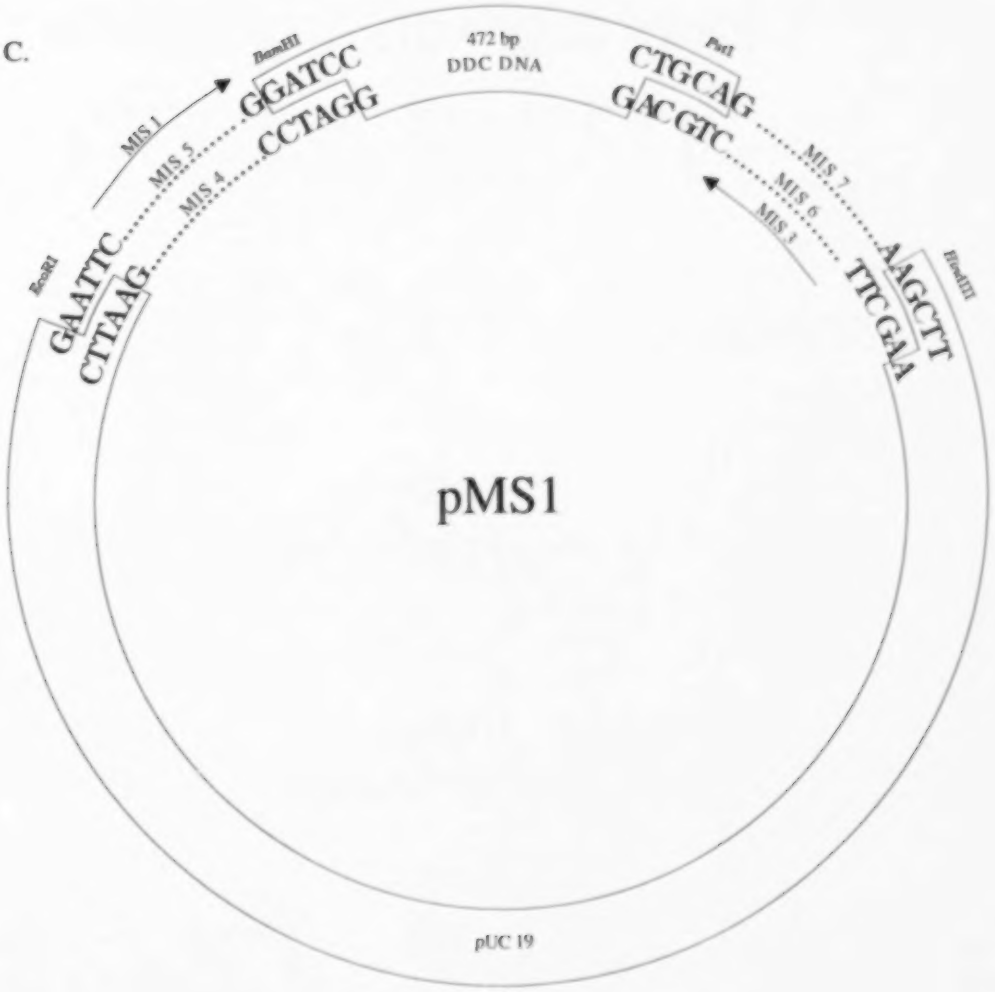
A.

MIS 1: 5' GCCAAGCGCACAGCAATCAG 3'
MIS 3: 5' ATGACTCGCTCGATGTCCTG 3'

B.



C.



Two oligonucleotides were constructed (Figure 14, A) which would prime the synthesis of a 370 bp product from the hypoderm specific cDNA (Figure 14, B). This choice ensured that the resulting product was distinguishable from any product templated by the neural specific cDNA and from the product templated by any contaminating genomic DNA that may have contaminated the original total RNA pool. The primers anneal to sequences in the first exon (MIS 1) and in the fourth exon (MIS 3), which span 2 exons and 3 introns. Tissue specific alternate splicing occurs in *Ddc* mRNA such that the second exon is included in the neural specific message and not in the hypodermal one. As a result, the hypodermal product would be 140 bp shorter than the neural product, and much shorter than the product primed on genomic DNA.

The competitor, pMS1, was designed such that MIS 1 and MIS 3 would prime a PCR product of 512 bp (Figure 14, C). The competitor plasmid was constructed using a 472 bp Ddc *Bam*HI / *Xho*I fragment spanning the third exon which was exised from ABG_1 (an M13 clone made by Ross Hodgetts) using *Bam*HI and *Pst*I. This was inserted into pUC 19 via two double stranded oligonucleotide linkers. One linker consisted of MIS 5, an oligonucleotide identical to MIS 1 but with extra nucleotides at either end to form parts of two different restriction sites (*Eco*RI and *Bam*HI), and MIS 4, its complement with the appropriate restriction site ends (Figure 14, C). Equimolar amounts of MIS 4 and MIS 5 were mixed, heated, and allowed to cool to room temperature to form this linker. The other linker was constructed in a similar manner. In this case, MIS 6 was identical to MIS 3 but with extra bases at either end to form part of the restriction sites *Pst*I and *Hin*dIII, and MIS 7 was its complement. The plasmid vector pUC 19 was cut with *Eco*RI and *Hin*dIII. The choice of restriction sites

insured that the 4 components of the competitor plasmid (the 472bp insert, the 2 linkers, and the vector) could be ligated in one step.

Reverse transcription reactions were carried out, using the MIS 3 oligonucleotide as a primer, on 100 ng pools of total RNA which had been extracted from the organisms of interest. The resulting cDNA was then subjected to competitive PCR in order to ascertain the quantity of hypodermal *Ddc* message present in the original total RNA pool. Competitive PCR was performed as described in "Materials and Methods". Eight reactions were set up; each contained a constant amount of cDNA but a differing amount of competitor plasmid. In most cases, the competitor added ranged from 0.00125 amol in the first reaction to 20.48 amol in the eighth reaction, quadrupling in each subsequent reaction mixture. Control reactions were performed to check for the presence of any possible contaminants: a reaction containing just the cDNA as a template, a reaction containing just the competitor as a template, a reaction containing RNA which had not been reverse transcribed, and a reaction to which no Taq polymerase had been added. The results of a typical experiment using RNA extracted from Canton S white prepupae is shown in Figure 15.

As in the DDC assays, RNA was extracted from the hemizygous BR-C mutant males of interest and from their wild type male siblings which were segregating in the same cross. This allowed for the quantity of hypodermal RNA, measured by competitive PCR in the mutant, to be expressed as a percentage of the quantity measured in the wild type. In order to minimize sources of error the following precautions were taken: the RNA from both the mutant and the wild type was reverse transcribed at the same time and for the same length of time. The resulting cDNA from both was subjected to competitive PCR in the same

PCR machine and in the same run. The products were then all run on the same gel. Three examples of typical results are shown in Figures 16 - 18. As a control for contamination, and to ensure that the reverse transcription reaction was successful, an aliquot of Canton S RNA was always reverse transcribed along with the mutant and wild type RNA from the strain of interest. This Canton S cDNA was subjected to PCR in the same run as the competitive PCR reactions of that strain.

Figure 15. An ethidium bromide stained 2% TAE agarose gel showing the products produced by competitive PCR when Canton S cDNA was used. The 512 bp product was templated by the competitor plasmid and the 370 bp product was templated by the hypodermal specific cDNA.

Lane designations are as follows:

L = 1 kb ladder (BRL)
-Taq = the PCR reaction contained all the substrates required for PCR (including cDNA template) except for Taq polymerase
-R.T. = the reaction contained everything required for PCR except that the only template added was RNA which had not been reverse transcribed.
0 = the reaction contained only cDNA as a template.
2 = the reaction contained 0.005 amol of competitor as well as cDNA as a template.
3 = the reaction contained 0.02 amol of competitor as well as cDNA as a template.
4 = the reaction contained 0.08 amol of competitor as well as cDNA as a template.
5 = the reaction contained 0.32 amol of competitor as well as cDNA as a template.
6 = the reaction contained 1.28 amol of competitor as well as cDNA as a template. This particular reaction did not work; possible reasons for this are addressed in the "Discussion".
7 = the reaction contained 5.12 amol of competitor as well as cDNA as a template.
8 = the reaction contained 20.48 amol of competitor as well as cDNA as a template
9 = the reaction contained 81.92 amol of competitor as well as cDNA as a template.
C. only = the reaction contained as its only template 5 amol of competitor.

L
-Taq
-R.T.
0
2
3
4
5
6
7
8
9
C. only
- 512 bp
- 370 bp

Figure 16. An ethidium bromide stained 2% TAE agarose gel showing the products produced by competitive PCR when the source of cDNA template is RNA extracted from ***rbp^4*** carrying males (top half of the gel) and from wild type males (bottom half of the gel). The 512 bp product was templated by the competitor plasmid and the 370 bp product was templated by the hypodermal specific cDNA. Lane designations are as follows:

L = 1 kb ladder (BRL)
0 = the PCR reaction contained no competitor
1 = the reaction contained 0.00125 amol of competitor
2 = the reaction contained 0.005 amol of competitor
3 = the reaction contained 0.02 amol of competitor
4 = the reaction contained 0.08 amol of competitor
5 = the reaction contained 0.32 amol of competitor
6 = the reaction contained 1.28 amol of competitor
7 = the reaction contained 5.12 amol of competitor
8 = the reaction contained 20.48 amol of competitor
CS = the reaction contained only Canton S cDNA as a template.

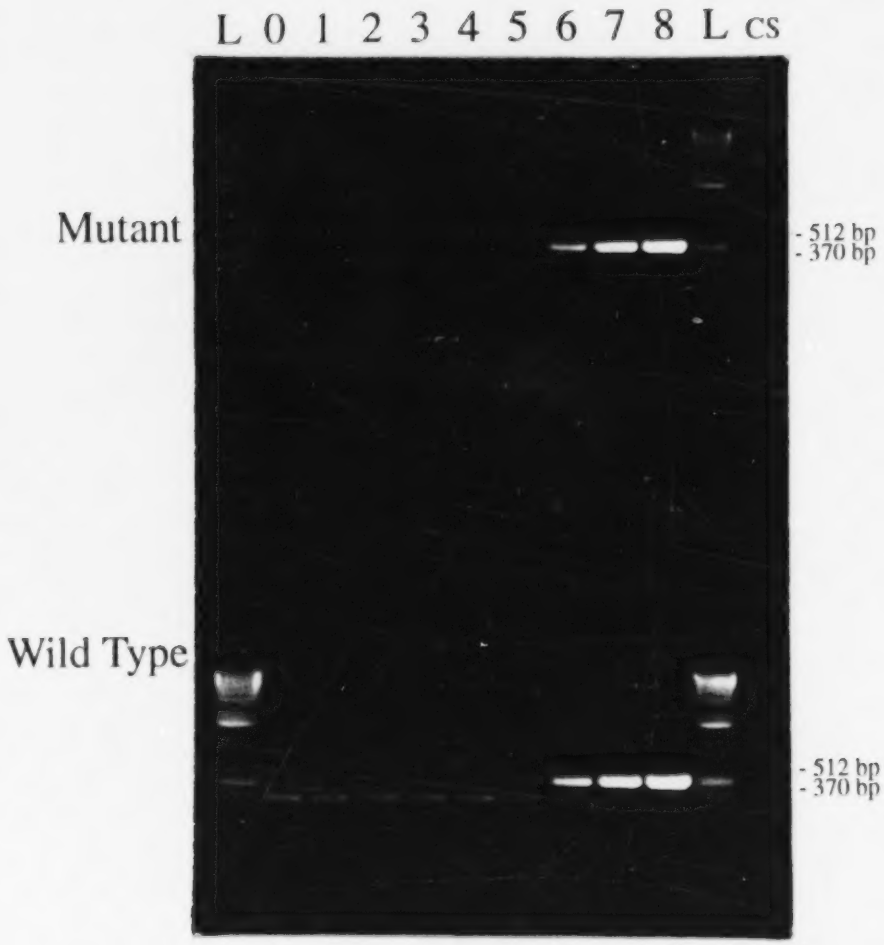

L 0 1 2 3 4 5 6 7 8 L cs
Mutant
Wild Type
- 512 bp
- 370 bp
- 512 bp
- 370 bp

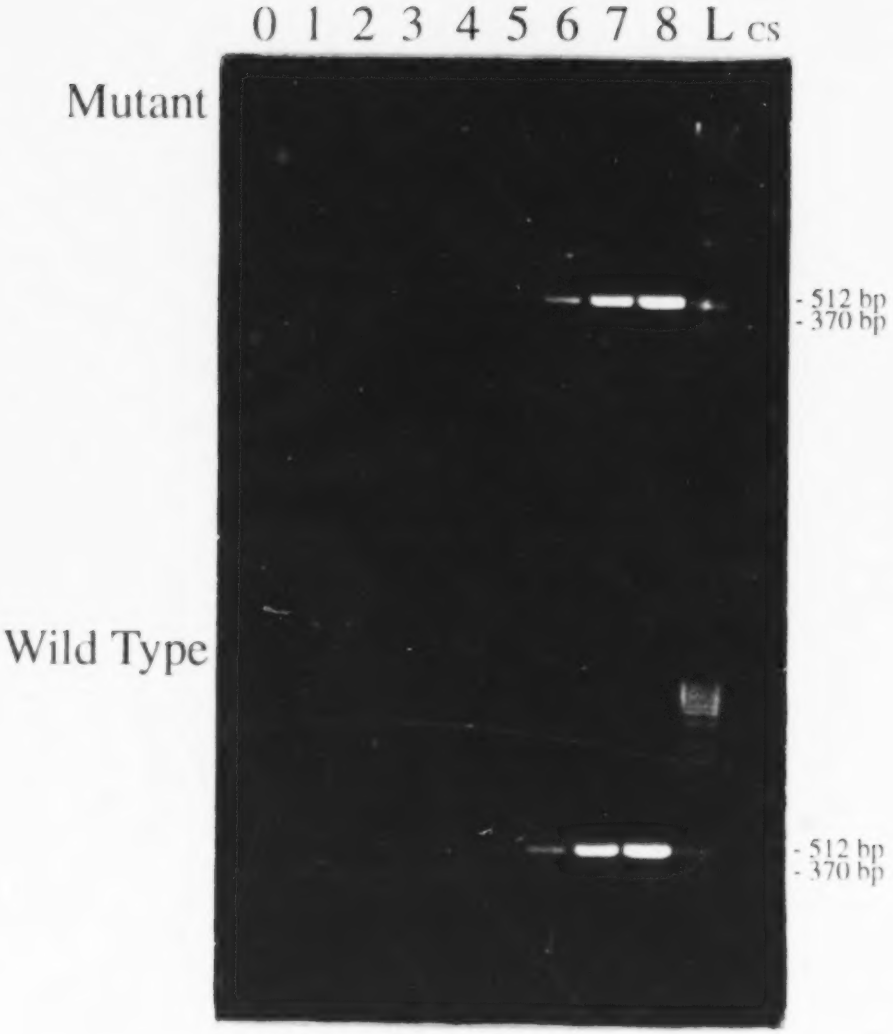
Figure 17. An ethidium bromide stained 2% TAE agarose gel showing the products produced by competitive PCR when the source of cDNA template is RNA extracted from br^{8} carrying males (top half of the gel) and from wild type males (bottom half of the gel). The 512 bp product was templated by the competitor plasmid and the 370 bp product was templated by the hypodermal specific cDNA. Lane designations are as follows:

L = 1 kb ladder (BRL)
0 = the PCR reaction contained no competitor
1 = the reaction contained 0.00125 amol of competitor
2 = the reaction contained 0.005 amol of competitor
3 = the reaction contained 0.02 amol of competitor
4 = the reaction contained 0.08 amol of competitor
5 = the reaction contained 0.32 amol of competitor
6 = the reaction contained 1.28 amol of competitor
7 = the reaction contained 5.12 amol of competitor
8 = the reaction contained 20.48 amol of competitor
CS = the reaction contained only Canton S cDNA as a template.

L 0 1 2 3 4 5 6 7 8 L cs
Mutant
- 512 bp
- 370 bp
Wild Type
- 512 bp
- 370 bp

Figure 18. An ethidium bromide stained 2% TAE agarose gel showing the products produced by competitive PCR when the source of cDNA template is RNA extracted from ***2Bab^5*** carrying males (top half of the gel) and from wild type males (bottom half of the gel). The 512 bp product was templated by the competitor plasmid and the 370 bp product was templated by the hypodermal specific cDNA. Lane designations are as follows:

L = 1 kb ladder (BRL)
0 = the PCR reaction contained no competitor
1 = the reaction contained 0.00125 amol of competitor
2 = the reaction contained 0.005 amol of competitor
3 = the reaction contained 0.02 amol of competitor
4 = the reaction contained 0.08 amol of competitor
5 = the reaction contained 0.32 amol of competitor
6 = the reaction contained 1.28 amol of competitor
7 = the reaction contained 5.12 amol of competitor
8 = the reaction contained 20.48 amol of competitor
CS = the reaction contained only Canton S cDNA as a template.


0 1 2 3 4 5 6 7 8 L cs
Mutant
- 512 bp
- 370 bp
Wild Type
- 512 bp
- 370 bp

In order to find the point where the ratio of cDNA PCR product to competitor PCR product is 1, the ethidium bromide stained bands on a gel were quantified using the Gel Print Toolbox version 2.0 computer program. This program plots the distribution of the intensity of staining of bands on a graph and integrates the area under each peak. As an example, the integral areas for each relevant peak (band) in the *2Bab*^{5} gel shown in Figure 18 are presented in Table 2. The integral areas for the competitor band were multiplied by 0.723 (370/512) to correct for the differences between its molecular weight and the experimental band. The ratio of the competitor product to the cDNA product was calculated and the log of this ratio was plotted against log(added competitor plasmid) (Figure 19). The point where the curve crosses the log(ratio)=0 line is the point were the amount of competitor added is equal to the amount of hypodermal specific cDNA reverse transcribed from the original 100 ng of total RNA. The value determined for the mutant was then expressed as a percentage of the value determined for the wild type control. In this example, the *2Bab*^{5} mutant strain exhibited 43% of the mRNA levels of the wild type. In the other two sample gels, shown in Figure 16 and Figure 17, *rbp*^{4} exhibited 87% and *br*^{8} exhibited 70% of wild type mRNA levels.

RNA was extracted from all strains in duplicate, and 1 (or 2, in the case of *br*^{28}) determination was done on each extraction. The results are shown in Table 3. One way analysis of variance showed a significant difference ($p<0.05$) between mutant strains but Duncan's multiple-range test (Snedecor and Cochran, 1980) resulted in no clear grouping of these differences because of the large variation among samples. However, all mutant BR-C alleles which showed a reduction in DDC activity also showed a reduction in steady-state mRNA levels.

A. 2Bab^5

Lane #	2	3	4	5	6	7	8
Intensity of competitor band	0	75	150	744	2521	7111	12087
Intensity of competitor band x 0.723	-	54	108	538	1822	5139	8735
Intensity of cDNA band	389	402	307	226	209	110	0
Competitor band / cDNA band	-	0.135	0.353	2.380	8.720	46.718	-
$\log\left(\frac{\text{Competitor band}}{\text{cDNA band}}\right)$	-	-0.870	-0.452	0.376	0.940	1.670	-

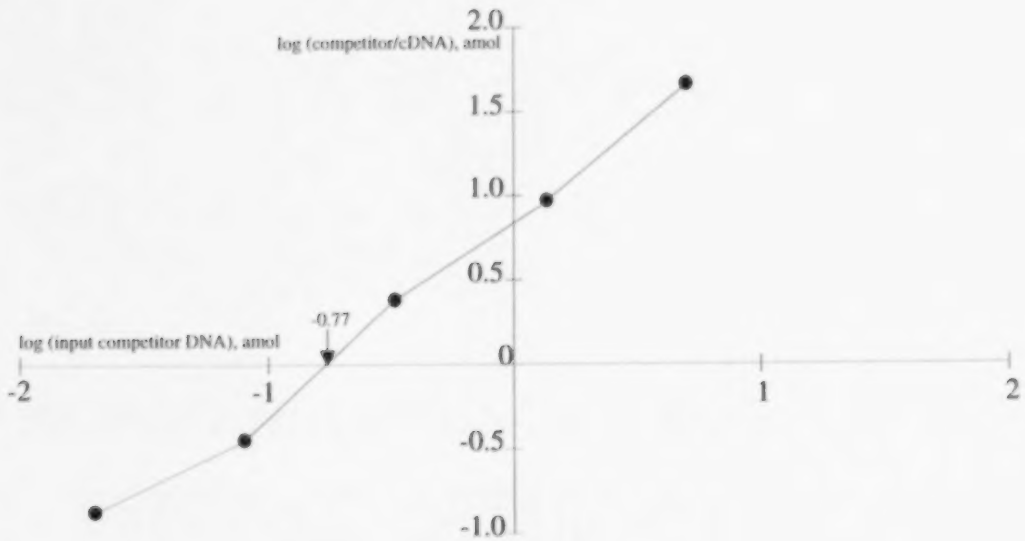
B. Wild Type

Lane #	2	3	4	5	6	7	8
Intensity of competitor band	0	58	176	791	2337	7065	12257
Intensity of competitor band x 0.723	-	42	127	572	1689	5106	8858
Intensity of cDNA band	967	897	780	683	530	280	152
Competitor band / cDNA band	-	0.047	0.163	0.837	3.186	17.850	58.276
$\log\left(\frac{\text{Competitor band}}{\text{cDNA band}}\right)$	-	-1.328	-0.788	-0.077	0.503	1.250	1.765

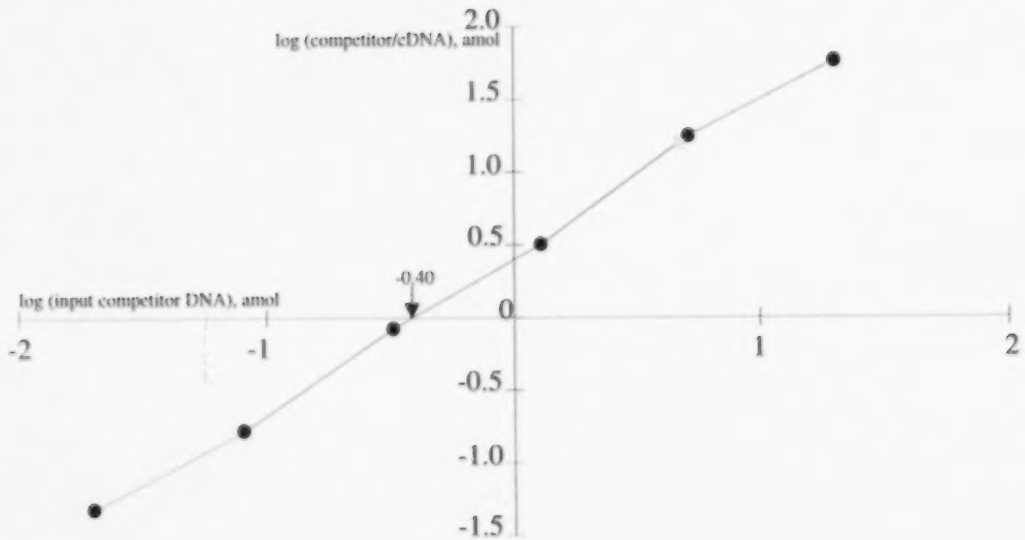
Table 2. Integral areas of peak DNA staining and calculations performed to obtain the data points which are plotted in Figure 19. This data was taken from the gel shown in Figure 18, (A) corresponds to the upper half of the gel where the cDNA template was reverse transcribed from *2Bab*^{5} RNA, and (B) corresponds to the lower half of the gel where the cDNA template was reverse transcribed from wild type RNA. All gels were analyzed in this manner.

Figure 19. Log plots of the competitor : cDNA ratios vs. input competitor calculated in Table 2. Graph (A) corresponds to table (A), and graph (B) corresponds to table (B). Arrows indicate the points where log(ratio) = 0, i.e. where the amount of competitor added is equal to the amount of hypodermal specific cDNA reverse transcribed from the original 100 ng of total RNA. The inverse log of -0.77 is 0.17 and the inverse log of -0.40 is 0.40. From this, the steady-state level of RNA in *2Bab*^{5} was calculated at 43% (0.17/0.40) of wild type.

A. 2Bab^5



B. Wild Type



Strain	Sample 1	Sample 2	Mean	S. D.
npr1^{7}	8	34	21.0	13.0
rbp^{4}	188	87	137.5	50.5
br^{5}	42	79	60.5	18.5
br^{8}	70	39	54.5	15.5
br^{28}	66; 22	8	32.0	24.7
br^{Aub}	103	141	122.0	19.0
2Bab^{5}	5	43	24.0	19.0

Table 3. Steady-state RNA levels in *BR-C*$^{-}$ males expressed as a percentage of wild type siblings segregating in the same cross. Organisms were collected on 2 separate occasions, the RNA extracted, reverse transcribed and subjected to competitive PCR (except for the first sample of RNA extracted from the *br*^{28} strain which was tested twice.). These results were then averaged and the standard deviation (S.D.) determined.

Discussion

Technical Considerations

The greatest technical difficulties were encountered in the competitive PCR procedures. The least of these was that occasionally a single reaction of a series failed to produce any of the expected PCR products (for an example, see Figure 15). This may have been caused by one of two possible problems: Taq polymerase was somehow omitted from the reaction mixture, or proper heat transfer from the PCR machine was not taking place. The first possibility is excluded in this case since Taq polymerase was added to a main master mixture from which mix required for all the reactions in a series was aliquoted. The problem was probably caused by faulty heat transfer. The PCR machine used was a Stratagene RoboCycler 40, which functions by rotating the tubes between metal temperature blocks, each at a different temperature. Heat transfer occurs from the metal well to the tube via oil placed in each well. If the tube had not been inserted properly into the machine such that it did not come in contact with the oil, or if the oil was low, heat transfer would not take place.

The second difficulty was the production of PCR products which had not been predicted. Early in this study, a band of about 250 bp consistently appeared in each reaction. It was sequenced and subjected to a homology search using GCG Version 6, FASTA Gen Bank data base at the National Research Council, but it was not found to be homologous to any known *Drosophila* sequences. Upon switching the reverse transcriptase in use from M-MLV (BRL) to AMV (Boehringer Mannheim), it disappeared. However, even when using AMV

reverse transcriptase, occasionally faint bands were present in a reaction which were larger than the bands expected from the hypodermal cDNA, the competitor plasmid (pMS1), or the neural cDNA. These were most likely a result of reverse transcription of precursors to mature *Ddc* mRNA as up to 3 different species of *Ddc* precursor have been detected in mature larvae (Spencer, 1987). Some reaction series contained faint bands whose size corresponded to the predicted product of the neural cDNA. Only reaction series resulting in the two expected products (i.e. the hypodermal cDNA product and the pMS1 product) were analyzed for hypodermal mRNA quantification purposes.

A recurrent problem was the contamination of all reactions in a series with competitor plasmid. When this occurred, every reaction, even control reactions to which no competitor plasmid had been added, contained an intense band whose size was equivalent to that of the pMS1 product. This problem was circumvented by replacing all solutions and mixes, by aliquoting all the new ones such that one aliquot was used per reaction series, and by using pipette tips designed to eliminate aerosols.

The greatest difficulty encountered was the large variation of quantified steady state RNA levels between duplicate RNA samples. The most straight forward explanation is that the RNA samples, extracted from separate collections of white prepupae, differed because of inconsistent developmental staging. This is not probable, however, as the organism staging for RNA extractions was carried out in an identical manner to the staging for DDC assays (see "Materials and Methods"), which do not show such variability between samples. The actual collection of organisms may have been a cause of error due to inconsistencies in the number of freezing and thawing rounds (which can result in RNA

degradation) to which each sample was subjected. The RNA extraction procedure itself might have been a source of error because of the difficulties in inhibiting RNase activity. These are unlikely to be the main sources of error, because duplicate tests on a single RNA sample (br^{28}, Table 3) also resulted in large variation. Another inconsistency could have arisen in the pipetting of the competitor plasmid dilution series, although precautions were taken (such as only using a recently calibrated P10 pipetman and ensuring that the competitor was pipetted in the largest possible volumes) to minimize this. Also, the presence of minute quantities of templates other than the hypodermal cDNA and pMS1, even if their products could not be detected, could have interfered with the competition dynamics between the two templates of interest. Another source of error lies in the graphing of the mutant to wild type ratios. All graphs were drawn by connecting the data points with a smooth curve. Although a straight line graph was expected, usually the lowest point (i.e. the point representing the lowest competitor product to cDNA product ratio) was not much lower than the next point in the series, indicating that the amount of cDNA product produced was maximized. So as not to skew the graph because of this spurious point, a curve was drawn rather than a best-fit line. However, even the slightest shift in the placement of the curve could result in 5% - 10% differences in the final calculations of the steady-state RNA levels.

Moreover, it was assumed throughout this work that a linear relationship exists between the amount of DNA present on a gel and the intensity of the signal detected by the Gel Print Toolbox. However, a calibration curve should have been constructed by measuring the intensities of differing known amounts of DNA for a wide range of values. This would have defined the interval of linearity

within which intensities should have been measured and might have reduced the variation. In addition, there may not be a linear relationship between the molecular weights of varying length double stranded DNA fragments and the intensity of their ethidium bromide staining, as was assumed in the calculations of this work. Again, a calibration curve measuring the intensity of staining versus fragment length may be a means of reducing any error incurred by this assumption. Finally, base composition may affect the intensity of staining since the dye may not have equal ability to intercalate all types of base pair sequences. In this case however, this is most likely not a factor since the differing sequences between the two PCR products have very similar G-C content: the competitor product has 41% and the cDNA product has 46% G-C content.

Dopa Decarboxylase Activity in *BR-C* mutants at the Second Moult

DDC activity at the hatch, first and second moults, and eclosion increases in response to a falling ecdysone titre rather than in response to a rising one as it does at pupariation (Figure 1) (Kraminsky *et al.*, 1980). This, coupled with the data of Estelle and Hodgetts (1984) who found a natural *Ddc* variant, Ddc^{+4}, which under-produces DDC at pupariation but over-produces at all other stages, indicates that the control of *Ddc* expression seems to be different at pupariation from all the other stages. In fact, the time lag between the peak of DDC activity and the ecdysone peak at the hatch, the first moult and the second moult seems to indicate control similar to the late genes of the Ashburner model (Figure 2) (Ashburner *et al.*, 1974), while the coincident increase of DDC activity with the

increasing ecdysone titre at pupariation is reminiscent of early gene control in the salivary glands. According to the model, late genes are positively regulated by early gene products. Three early genes, visualized as early puffs in the salivary glands, have been characterized: *E74* (Burtis *et al.*, 1990), *E75* (Segraves and Hogness, 1990) and *BR-C* (Chao and Guild, 1986; DiBello *et al.*,1991). This study has shown that mutations in alleles of *BR-C* have no effect on DDC expression at the second moult, while certain alleles have a significant effect on DDC production at pupariation (Figures 9-13 and Appendix 1). These data support the hypothesis that *Ddc* regulation is under two different control mechanisms at these two stages. It would be interesting to test DDC activity in *E74* and *E75* mutant backgrounds at both of these developmental stages.

Dopa Decarboxylase Activity in White Prepupal Brains of *BR-C* Mutants

Not only does *Ddc* appear to be under differential temporal control, but it also shows tissue specificity. Ddc is expressed in the hypoderm, where over 90% of the total DDC activity in an organism resides (Lunan and Mitchell, 1969), and in the CNS, where the remaining DDC activity is found (Scholnick *et al.*, 1986). While DDC activity in the hypoderm fluctuates greatly over the course of development, its activity in the CNS remains fairly constant (Scholnick *et al.*, 1986). Scholnick *et al.* (1986) confirmed that control of Ddc expression in the two tissues could be separated. They discovered sequences in the region upstream of the transcription start site that, when mutated, resulted in a decrease in neural DDC activity but not in hypodermal DDC activity. Our studies have strengthened

their findings. Even though several mutant alleles of BR-C, including the null allele *npr1*^{7}, showed large reductions of DDC activity at pupariation, no effect was found on brains of *npr1*^{7} animals at this stage. This indicates that alleles of *BR-C* must only be involved in *Ddc* regulation in the hypoderm and not in the CNS at pupariation. It is interesting to note that BR-C function does seem to be required in the CNS at a later stage: metamorphosis (Restifo and White, 1991). However, in this case it is involved in the proper organization of the optic lobes (i.e. cell migration) and probably has no influence on *Ddc* expression.

Dopa Decarboxylase Expression in *BR-C* Mutants at Pupariation

As stated earlier, several *BR-C* alleles cause a severe reduction in DDC activity at pupariation (Figures 9 - 13 and Appendix 1). Clark *et al.* (1986) found that, although *Ddc* is induced directly by ecdysone, it requires protein synthesis for full induction. Thus *Ddc* regulation at this stage is complex: not only does it exhibit control similar to the early genes in the salivary glands, but it seems to share a component of its control with the late genes. Our results confirm our hypothesis that, like the salivary late genes, an early gene product mediates full DDC induction at pupariation.

A null allele for the *BR-C* locus, *npr1*^{7}, showed the most severe result (12.5% of wild type DDC activity, Figure 9). This indicates that *BR-C* is required for DDC induction at pupariation. It does not, however, reveal whether or not *BR-C* controls *Ddc* directly. Animals carrying the *npr1*^{7} mutation never pupariate (Kiss *et al.*, 1988). Thus it seems that *BR-C* is necessary to provide the signal to

begin pupariation, *Ddc* being one of the many genes turned on in the ecdysone-induced gene cascade. *Ddc* may be turned on directly by a *BR-C* gene product or there may be several intermediary genes which transfer the signal from *BR-C* to *Ddc*. This latter possibility is rendered unlikely by the DNA binding studies described below.

The allele $npr1^{7}$ falls into the non-complementing complementation group, meaning that it contains no working subfunctions of *BR-C*. In order to narrow down which subfunctions are involved in *Ddc* control, alleles of the other four complementation groups (Figure 3) were tested for DDC activity. Two alleles of *2Bc* were tested and found to have no affect (Figure 10), suggesting that this function plays no role in *Ddc* control. Also found to have no affect was the allele rbp^{4} , (Figure 11), again implying that this function plays no role in *Ddc* regulation. Three alleles (of four tested) of the *br* complementation group did show a reduction in DDC activity (Figure 12) and one allele of *2Bab* (of four tested) did as well (Figure 13). This data strongly suggests that the *br* subfunction is the one required for full DDC induction at pupariation.

DDC activity in the *br* alleles was reduced by 46% - 79% of wild type activity (Figure 12). A conclusion that might be drawn from this is that the *br* alleles range in their severity from br^{8}, the weakest, through br^{28} to br^{5}, the strongest. In the literature (Kiss *et al.*, 1988; DiBello *et al.*, 1991), these three alleles are described as being amorphs and having very similar phenotypes. This study, then, has provided a means by which to distinguish them phenotypically. The only allele of the three which has been mapped is br^{28}: it is a P-element insertion in the Z2 zinc finger pair of *BR-C* (Sampedro et al., 1989) (Figure 4). It follows that Z2 may be the mediator of the *br* function of *BR-C*. The allele br^{Aub}

is a viable allele and a weak hypomorph (C. Bayer, pers. comm.), which distinguishes it from the three lethals described above. Another distinguishing factor is that it does not cause a decrease in DDC activity at pupariation, but instead, a slight increase. Perhaps this allele is not a hypomorph, but rather a weak neomorph, as its *br* function is capable of up-regulating DDC activity at this stage.

The *2Bab* complementation group does not complement either *br* or *rbp* alleles (Kiss *et al.*, 1988; C. Bayer, pers. comm.). Our data shows that only one allele, $2Bab^5$ (Figure 13), mediates a decrease in DDC activity at pupariation, while the other three tested do not. This decrease is on an order of magnitude similar to that of the lethal *br* alleles: 69%. Kiss *et al.* (1988) hypothesized that *br* and *rbp* alleles may actually be members of the same complementation group and affect the same *BR-C* function. However, our data produce further evidence (beyond that previously described in the "Introduction") to suggest that *br* and *rpb* do indeed represent different *BR-C* functions in that *br* alleles cause a decrease in DDC activity at pupariation while rbp^4 does not. In addition, the *br* and *rbp* functions seem to be distinguishable amoung *2Bab* alleles. The allele $2Bab^5$ seems to affect *Ddc* in a manner akin to the lethal *br* alleles, while $2Bab^1$, $2Bab^3$ and $2Bab^6$ have no effect, like rbp^4.

The data collected on steady-state *Ddc* mRNA levels in *BR-C* mutants using competitive PCR is difficult to analyze because of the large variation between samples (Table 3). The most that can be drawn from this data is that those alleles that have no effect on DDC activity (rpb^4 and br^{Aub}) also appear to have no significant effect on steady-state *Ddc* mRNA levels, while those that do show a reduction in DDC activity also show a reduction in steady-state mRNA

levels. This suggests that the *br* function may be involved in preventing degradation of *Ddc* mRNA, or, a more likely hypothesis, it may be involved in the transcriptional regulation of *Ddc*. It is interesting to note that Clark *et al.* (1986) found that *Ddc* transcripts were reduced approximately 75% in the absence of protein synthesis, and these studies show between 40%- 80% reduction in steady-state *Ddc* mRNA in the *BR-C* alleles of interest. Perhaps, then, the *BR-C* protein product containing the *br* function is all that is required for full *Ddc* induction at pupariation.

Z2 Binds to the First Intron of *Ddc*

Dissecting the control elements of Ddc regulation has proven to be a very difficult task. All the elements necessary for wild type DDC expression are contained within a 7.5 kb *Pst* I fragment which contains 2.8 kb of 5' flank DNA, 4.0 kb of the gene itself and 0.7 kb of 3' flank DNA (Scholnick *et al.*, 1983). Hirsh *et al.* (1986) found that although sequences from -209 to -24 relative to the transcription start site are required for normally regulated gene expression, some components of normal developmental expression (such as induction of DDC at pupariation) are retained in the absence of these sequences. This points to the existence of regulatory sequences lying downstream of the transcription start site. Furthermore, transformation experiments with fusion genes containing upstream Canton S *Ddc* DNA and downstream Ddc^{+4} DNA (and vice versa) suggested that differences in *Ddc* control between the two fusion constructs were mediated by the downstream sequences (Patel, 1992).

Since *Ddc* expression at pupariation seems to be regulated in part by the *br* function of *BR-C*, and since the *br* function may be correlated with the Z2 zinc finger pair, we hypothesized that Z2 may bind to regulatory regions of *Ddc*. Recent work in our laboratory has shown that a recombinant protein, containing the Z2 zinc finger pair from *BR-C* (provided by K. Crossgrove), binds to the first intron of *Ddc* (W. Clark, pers. comm.). In fact, footprinting studies have revealed that Z2 binds to three A/T rich regions within this intron (W. Clark, pers. comm.). This confirms the data of Hirsh *et al*. (1986) and Patel (1992) which implied that regions downstream of the transcription start site may be involved in *Ddc* regulation.

The natural variant, Ddc^{+4}, is an underproducer of DDC at pupariation but an overproducer at all other stages (Estelle and Hodgetts, 1984). It contains polymorphic sites in the first intron that have not as yet been sequenced (Estelle and Hodgetts, 1984). Footprinting studies of Z2 onto the first intron of Ddc^{+4} might be anticipated to exhibit a different binding pattern than that of Canton S *Ddc*, shedding further light on the control elements of *Ddc*. However, DDC assays on br^5 mutants in a Ddc^{+4} background revealed a similar reduction in DDC activity as that seen in a wild type background (70.5% ± 0.5%, data not shown) indicating that Ddc^{+4} polymorphisms are most likely not associated with *BR-C* control of *Ddc*. This is intriguing as it implies that *Ddc* expression at pupariation may be regulated by more factors than ecdysone and *BR-C* and that even greater complexities are involved in its developmental gene expression.

Further Studies

To dissect further the control of *Ddc*, as alluded to earlier in this discussion, it may be useful to test the effect, if any, of mutations in the early genes *E74* and *E75* on DDC production. This study has shown that mutations in BR-C have no affect on DDC activity at the second moult. However, the time lag between DDC production and the ecdysone peak at this stage (as well as at the hatch and the first moult) is similar to that of late genes in the salivary glands at pupariation. Perhaps protein synthesis is required for DDC production at the second moult, and if that is the case, perhaps the protein products of *E74* or *E75* (rather than those of *BR-C*) mediate Ddc induction at that stage. If this proved to be the case, then not only could the Ashburner model (Ashburner *et al.*, 1974) be extended to tissues other than the salivary glands, but also to developmental stages other than pupariation.

It would also be interesting to test the effects of *E74* and *E75* mutants on DDC production at pupariation. This study strongly suggests that the *br* function of *BR-C* is all that is necessary to mediate the full induction of DDC. However, the observation that DDC activity in br^5 mutants is not reduced in a Ddc^{+4} background any more than it in a Canton S background, indicates that whatever causes the natural reduction in DDC in Ddc^{+4} at this stage is separate from the influence of *BR-C*.

As it is only a hypothesis that the Z2 zinc finger pair of *BR-C* corresponds to the *br* subfunction, further footprinting studies on *Ddc* ought to be done. If the hypothesis is correct, Z1 and Z3 carrying recombinant proteins should not bind to *Ddc*. These experiments are currently being carried out by L. Von Kalm.

Recombinant proteins containing the Z4 zinc finger pair might bind as Z4 transcripts have been detected in all tissues tested to date (C. Bayer, pers. comm.). It thought to act as a 'helper' binding protein (C. Bayer, pers. comm.), that is, it may form heterodimers with other *BR-C* DNA binding proteins and thus assists in their binding.. Another important experiment is to test which zinc fingers are expressed in the hypoderm at pupariation. These studies are currently underway in our laboratory using Northern Blot analysis; a possible alternative would be to do reverse transcription PCR as described in Huet *et al.* (1993) to detect the different zinc finger domains of *BR-C*. This technique is similar to the competitive PCR described in this study, except that it only detects the presence or absence of transcripts rather than quantifies them. As in the footprinting studies, if Z2 is the sole mediator of the *br* function, we would expect to find Z2 transcripts in the hypoderm with perhaps some Z4 transcripts.

Conclusions

This study has shown that the *br* function of the early gene, *BR-C*, is essential for full *Ddc* expression in the hypoderm at pupariation, but not at either the second moult or in the CNS. Thus it can be concluded that regulation of Ddc expression is indeed under different control mechanisms at these two stages and in these two tissues. The *br* function of *BR-C* has been shown to be required in wing discs (Gotwals and Fristrom, 1991) and in the optic lobes (Restifo and White, 1991) at metamorphosis. It also seems to play only a minor role in the salivary glands at pupariation (Karim and Thummel, 1993). In addition to these roles, we

have found that it is required in the hypoderm at pupariation. Karim and Thummel (1993) have demonstrated that wild type *BR-C* is necessary for full induction of the three ecdysone-inducible early genes: *BR-C, E74* and *E75* at pupariation, which is very similar to the control of *Ddc* induction as described in this work. Thus, *Ddc* can be classified as an early gene, if a more inclusive definition of an early gene than that originally used by Ashburner *et al*. (1974) is adopted. The Ashburner model, developed to describe the patterns of hormone induced gene expression in the salivary glands, may be a more general paradigm of ecdysone-inducible gene cascades in other tissues. However, it remains to be seen if the set of early genes visualized in the salivary glands is common to all target tissues of the hormone.

Bibliography

Andres AJ; Fletcher JC; Karim FD; Thummel CS (1993): Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* in press.

Andres AJ; Thummel CS (1992): Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. *T.I.G.* **8:** 132-138.

Ashburner M; Chihara C; Meltzer P; Richards G (1974): Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbour Symp. Quant. Biol.* **38:** 655-662.

Belyaeva ES; Aizenzon MG; Semeshin VF; Kiss I; Koczya M; Baritcheva M; Gorelova TD; Zhimulev IF (1980): Cytogenetic analysis of of the 2B3-4-2B11 region of the X chromosome of *Drosophila melanogaster*. I. Cytology of the region and mutant complementation groups. *Chromosoma.* **81:** 281-306.

Belyaeva ES; Vlassova IE; Biyasheva ZM; Kakpakov VT; Richards G; Zhimulev IF (1981): Cytogenetic analysis of the 2B3-4-2B11 region of the X chromosome of *Drosophila melanogaster*. II. Changes in 20-OH ecdysone puffing caused by genetic defects of puff 2B5. *Chromosoma.* **84:** 207-219.

Burtis KC; Thummel CS; Jones CW; Karim FD; Hogness DS (1990): The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell.* **61:** 85-99.

Chao AT; Guild GM (1986): Molecular analysis of the ecdysterone-inducible 2B5 "early" puff in *Drosphila melanogaster*. *EMBO J.* **5:** 143-150.

Clark WC; Doctor J; Fristrom JW; Hodgetts RB (1986): Differential responses of the dopa decarboxylase gene to 20-OH ecdysone in *Drosophila melanogaster*. *Dev. Biol.* **114:** 141-150.

DiBello PR; Withers DA; Bayer CA; Fristrom JW; Guild GM (1991): The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics.* **129:** 385-397.

Estelle MA; Hodgetts RB (1984): Insertion polymorphisms may cause stage specific variation in mRNA levels for *dopa decarboxylase* in *Drosophila*. *Mol. Gen. Genet.* **195:** 442-451.

Evans RM; Hollenberg SM (1988): Zinc fingers: guilt by association. *Cell.* **52:** 1-3.

Gietz RD; Hodgetts RB (1985): An analysis of *dopa* decarboxylase expression during embryogenesis in *Drosophila melanogaster*. Dev. Biol. **107:** 142-155.

Gilliland G; Perrin S; Blanchard K; Bunn HF (1990): Analysis of cytokine mRNA and DNA: detection and quantitation by competitve polymerase reaction. *Proc. Natl. Acad. Sci. USA.* **37:** 2725-2729.

Gotwals PJ; Fristrom JW (1991): Three neighbouring genes interact with the *Broad Complex* and the *Stubble-stubbloid* locus to affect imaginal disc morphogenesis in *Drosophila. Genetics* **127:** 747-759.

Guay PS; Guild GM (1991): The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a *Broad Complex* early gene regulates intermolt and late gene transcription. *Genetics* **129:**169-175.

Hirsh J; Davidson N (1981): Isolation and characterization of the *dopa* decarboxylase gene of *Drosophila melanogaster*. *Mol. Cell. Biol.* **1:** 475-485.

Hirsh J; Morgan BA; Scholnick SB (1986): Delimiting regulatory sequences of the *Drosophila melanogaster Ddc* gene. *Mol. Cell. Biol.* **6:** 4548-4557.

Huet F; Ruiz C; Richards G (1993): Puffs and PCR: the *in vivo* dynamics of early gene expression during ecdysone responses in *Drosophila. Devel.* **118:** 613-627.

Karim FD; Thummel CS (1992): Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11:** 4083-4093.

Karim FD; Guild GM; Thummel CS (1993): The Drosophila *Broad Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Devel.* in press.

Karlson P; Sekeris CE (1962): Zum tyrosinstoffwechsel der insekten. IX. Kontrolle des turosin stoffwechsels durch ecdyson. *Biochem. Biophys. Acta.* **63:** 489-495.

Kiss I; Beaton AH; Tardiff J; Fristrom D; Fristrom JW (1988): Interactions and developmental effects of mutations in the *Broad Complex* of *Drosophila melanogaster*. *Genetics* **118:** 247-259.

Konrad KD; Marsh JL (1987): Developmental expression and spatial distribution of *dopa* decarboxylase in *Drosophila*. *Dev. Biol.* **122:** 172-185.

Kraminsky GP; Clark WC; Estelle MA; Gietz RD; Sage BA; O'Connor JD; Hodgetts RB (1980): Induction of translatable mRNA for *dopa* decarboxylase in *Drosophila*: An early response to ecdysterone. *Proc. Natl. Acad. Sci. USA*. **77:** 4175-4179.

Lindsley D; Zimm GG (1992): The Genome of *Drosophila melanogaster*. Academic press, Toronto, Ontario.

Lunan KD; Mitchell HK (1969): The metabolism of tyrosine-o-phosphate in *Drosophila. Arch. Biochem. Biophys.* **132:**450-456.

Marsh JL; Wright TRF (1980): Developmental relationship between *dopa* decarboxylase, dopamine acetyltransferase, and ecdysone in *Drosophila*. *Dev. Biol.* **80:** 379-387.

Melton DA; Krieg PA; Rebagliati MR; Maniatis T; Zinn K; Green MR (1984): efficient i*in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12:** 7035-7055.

Miller J; McLachlan AD; Klug A (1985): Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J.* **4:** 1609-1614.

Myerowitz EM; Raghavan KV; Mathers PH; Roark M (1987): How Drosophila larvae make glue: control of Sgs-3 gene expression. *T.I.G.* **3:** 288-293.

Nash DN; Bell JB (1968): Larval age and the pattern of DNA synthesis in polytene chromosomes. *Can. J. Genet. Cytol.* **10:** 82-92.

Nelson RJ; Odell GM; Christiansen AE; Laird CD (1991): Hormonal control of gene expression: Interactions between two *trans*-acting regulators in Drosophila. *Dev. Biol.* **144:** 152-166.

Patel MS (1992): The effects of promoter mutations on the developmental expression of the *dopa* decarboxylase gene in *Drosophila melanogaster*. M.Sc Thesis, University of Alberta (Edmonton).

Restifo LL; White K (1991): Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in *Drosophila*. *Dev. Biol.* **148:** 174-194.

Restifo LL; White K (1992): Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle and gut. *Wilhelm Roux's Arch. Dev. Biol.* **201:** 221-234.

Sambrook J; Fritsch EF; Maniatis T (1989): Molecular Cloning, A Laboratory Manual. Second Edition. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Sampedro J; Galceran J; Izquierdo M (1989): Mutation mapping of the 2B5 ecdysone locus in *Drosophila melanogaster* reveals a long-distance controlling element. *Mol. Cell. Biol.* **9:** 3588-3591.

Scholnick SB; Bray SJ; Morgan BA; McCormick CA; Hirsh J (1986): CNS and hypoderm regulatory elements of the *Drosophila melanogaster dopa* decarboxylase gene. *Science* **234:** 998-1002.

Scholnick SB; Morgan BA; Hirsh J (1983): The cloned *dopa* decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell* **34:** 37-45.

Segraves WA; Hogness DS (1990): The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4:** 204-219.

Snedecor GW; Cochran WG (1980): Statistical Methods. Seventh Edition. Iowa State University, Ames, Iowa.

Spencer CA (1987): Molecular genetic studies on the *dopa* decarboxylase gene in *Drosophila melanogaster*. Ph. D. Thesis, University of Alberta (Edmonton).

Walter MF; Black BC; Afshar G; Kermabon A-Y; Wright TRF; Biessmann H (1991): Temporal and spatial expression of the *yellow* gene in correlation with cuticle formation and *dopa* decarboxylase activity in *Drosophila* development. *Dev. Biol.* **147:** 32-45.

Wright TRF (1987): The genetic and molecular organization of the dense cluster of functionally related, vital genes in the *dopa* decarboxylase region of the *Drosophila melanogaster* genome. Fourteenth Edition. (Series Ed: Hennig W. Results and Problems in Cell Differentiation. Sructure and Function of Eukaryotic Chromosomes.) pp. 95-120. Springer-Verlag, Berlin, Heidelberg.

Zhimulev IF; Vlassova IE; Belyaeva ES (1982): Cytogenetic analysis of the 2B3-4-2B11 region of the X chromosome of *Drosophila melanogaster*. III. Puffing disturbance in salivary gland chromosomes of homozygotes for the mutation *l(1)pp1t10*. *Chromosoma.* **85:** 659-672.

Appendix 1. DDC activity in *BR-C*$^{-}$ males expressed as a percentage of the activity of wild type siblings segregating in the same cross, and shown at two stages of development: A) the second moult, and B) pupariation. Organisms from each strain were collected on 2 or 3 separate occasions (except for br^{5} at the second moult). These results were then averaged and the standard deviation (S.D.) determined.

A. Second Moult

Strain	Sample 1	Sample 2	Mean	S. D.
npr1^{7}	96	126	111.0	15.0
2Bc^{1}	99	93	96.0	3.0
2Bc^{2}	94	105	99.5	5.5
rbp^{4}	98	101	99.5	1.5
br^{5}	94	---	94.0	0
br^{8}	85	94	89.5	4.5
br^{28}	115	102	108.5	6.5
br^{Aub}	124	110	117.0	7.0
2Bab^{1}	105	89	97.0	8.0
2Bab^{3}	116	102	109.0	7.0
2Bab^{5}	125	102	113.5	11.5
2Bab^{6}	94	109	101.5	6.5

B. Pupariation

Strain	Sample1	Sample2	Sample 3	Mean	S. D.
npr1^{7}	12	13	---	12.5	0.5
2Bc^{1}	90	86	94	90.0	3.3
2Bc^{2}	92	90	---	91.0	1.0
rbp^{4}	100	105	---	102.5	2.5
br^{5}	23	20	---	21.5	1.5
br^{8}	54	54	---	54.0	0
br^{28}	29	29	---	29.0	0
br^{Aub}	124	125	---	124.5	0.5
2Bab^{1}	105	87	106	99.3	8.7
2Bab^{3}	103	82	88	91.0	8.8
2Bab^{5}	35	28	---	31.5	3.5
2Bab^{6}	107	101	---	104.0	3.0